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EFFECT OF GLUCAGON AND EPINEPHRINE ON REGIONAL METABOLISM OF GLUCOSE, PYRUVATE, LACTATE, AND CITRATE IN NORMAL CONSCIOUS DOGS[1]

DOROTHY H. HENNEMAN[2] AND WILLIAM C. SHOEMAKER[3]

Departments of Anesthesia and Surgery, Peter Bent Brigham Hospital and Harvard Medical School, Boston, Massachusetts; Department of Medicine, Seton Hall College of Medicine, Jersey City, New Jersey, and the Department of Experimental Surgery, Michael Reese Hospital and Medical Center, Chicago, Illinois

ABSTRACT

Simultaneous arterio-venous concentration gradients and blood flow were measured across the liver and hindquarters following the administration of glucagon and epinephrine in postabsorptive and fasted, conscious dogs. In the postabsorptive dog glucagon increased the production of glucose and the uptake of pyruvate, lactate and citrate by the liver. Glucagon also increased the net production of lactate and citrate by the hindquarters; hindquarter uptake of pyruvate increased slightly. Hepatic blood flow increased and hind limb blood flow decreased after glucagon administration. The data indicate considerable recycling of the organic acids. Fasting depressed the effects of glucagon on regional glucose metabolism and regional blood flow but not on regional pyruvate, lactate or citrate metabolism. Thus, the effects of glucagon on peripheral glucose uptake and blood flow appear to be related to changes in blood glucose levels while the effects of both hepatic and peripheral metabolism of the other compounds measured do not.

In contrast to glucagon, epinephrine decreased hindquarter uptake of pyruvate and glucose. However, as with glucagon, small doses of epinephrine increased the net peripheral production of pyruvate and lactate; at the same time it also increased their uptake by the liver. In general, epinephrine, like glucagon, affected the homeostatic balance between liver and peripheral tissues by increasing the hepatic glycogenolysis as well as accelerating the circulation of the components of the Cori cycle.

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[2] Present address: Seton Hall College of Medicine, Jersey City, New Jersey.

[3] Present address: Michael Reese Hospital and Medical Center, Chicago, Illinois.

INTRODUCTION

UNCERTAINTY as to the effect of glucagon on peripheral glucose metabolism has resulted in part from measurements of arterio-venous concentration gradients without simultaneous measurements of blood flow (1, 2). The relation of increased circulating glucose levels *per se* to changes of tissue glucose utilization has not been adequately determined in the intact animal. The present studies were undertaken to measure simultaneously arterio-venous concentration gradients and blood flow across the liver, hindquarters, and abdominal viscera exclusive of liver in the postabsorptive and fasted, nonanesthetized dogs. Glucagon administered to the fasted (i.e., glycogen depleted) animal produces minimal or insignificant increases in hepatic glucose production with little or no rise in circulating blood glucose levels (3). It was anticipated, therefore, that comparison of the effects of glucagon on peripheral carbohydrate metabolism under both fed and fasting conditions might provide information on whether the peripheral action of glucagon was a direct one.

The present studies indicate that the response to glucagon in the intact animal may vary from one peripheral area to another; hence changes in isolated tissues may not reflect the over-all metabolic picture. Moreover, the effect of glucagon on pyruvate, lactate and citrate metabolism in our studies do not appear to be solely dependent upon the circulating blood glucose levels.

METHODS

Hepatic metabolism was studied after catheterization of the major hepatic vessels with chronic indwelling plastic tubing according to the technique of Shoemaker, *et al.* (4). Two to eight days after insertion of the catheters the dogs were studied while in a nonsedated, conscious state. Two types of dogs were compared: the first group, in good nutritional state, was fasted overnight (approximately 17 hours); the second group was fasted for 5 days, but allowed water *ad libitum*. Hepatic blood flow was measured with a modified bromsulphalein clearance technique (5, 6). Prior to the experiment, the dogs were placed in a Pavlov stand. After a period of time allowed for the equilibration of the bromsulphalein, simultaneous samples of arterial, portal and hepatic venous blood were withdrawn at intervals during a control period and after the experimental variable. An equivalent volume of blood from a donor dog was used to replace the sampled blood. The rate of hepatic uptake or output of a given substance was calculated by multiplying the portal-hepatic concentration gradient by the measured hepatic blood flow. Concentration gradients between the splenic artery and portal vein reflect the metabolism of the area drained by the portal vein which includes most of the gastrointestinal tract, lymph nodes, pancreas, and omentum. For the purposes of the present study this region is referred to as "nonhepatic splanchnic area" and its gradient as "splanchnic gradient."

Hindquarter metabolism was studied by sampling from cannulae or catheters placed in the distal inferior vena cava and carotid artery together with concomitant direct measurement of hindquarter blood flow. Hindquarter concentration gradients were obtained: 1) without flow measurements in nonanesthetized dogs, and 2) with light pentobarbital anesthesia where hindquarter blood flow was measured directly using a graduated cylinder and chronometer (7). The changes in concentration gradients observed with and without this light anesthesia were not significantly different.

TABLE 1. RESTING, REGIONAL METABOLISM IN NORMAL, CONSCIOUS DOGS

Organ	No. of dogs	No. of samples	Glucose mg./min.		Pyruvate mg./min.		Lactate mg./min.		Citrate mg./min.	
			Mean	SE	Mean	SE	Mean	SE	Mean	SE
Liver	13	20–68	− 74	18	1.09	0.21	5.0	0.4	1.5	0.23
	4		−180	9.5	3.2	0.46	12.5	1.3	5.9	0.22
Hindquarters	7	20	28.4	5.3	0.78	0.68	−2.6	1.0	−1.26	0.19

The rate (mg./min.) of uptake (positive) or output (negative) calculated by multiplying the arterio-venous concentration gradient by the simultaneously measured blood flow for each individual sample. Mean of the individual rates and standard error of the mean(SE) are given.

The chemical techniques used for measurement of glucose, pyruvate, lactate and citrate were the same as reported previously (8).

Intravenous glucagon[4] (.02 mg./kg.) or epinephrine[5] (1 to 5 μg/kg.) were administered as a single, rapid injection into a peripheral vein.

RESULTS

Resting Hepatic Metabolism of Postabsorptive, Conscious Dogs

Rates of production or uptake of glucose, pyruvate, lactate and citrate in nonanesthetized dogs are shown in Table 1. The average weight of the first group of dogs was 15 kg.; the second group weighed an average of 20 kg. If measurements of the rate of metabolism of the various carbohydrates could be extrapolated from the period of observation to a 24-hour period, as has been done in the case of renal metabolic measurements (9), then the calculated resting hepatic uptake of pyruvate, lactate and citrate varied from 11 to 31 gm. per day. The hepatic uptake of citrate alone varied from 2.2 to 8.5 gm./24 hours.

Effects of Glucagon in Normal Dogs

Blood Flow Changes

The effect of glucagon on hepatic blood flow has been reported previously (3); in the present series of animals the observed increase was maximal 10 to 20 minutes after glucagon and coincided with the maximal increase in hepatic glucose production (Figs. 1, 2). A simultaneous decrease in hindquarter blood flow was observed in this series. Ten to 20 minutes after glucagon the hindquarter blood flow reached its lowest point, which approximately coincided with the period of maximally increased circulating glucose levels and with the maximal rate of peripheral glucose uptake (Figs. 1, 2).

Glucose Changes

An increase in net hepatic glucose production in dogs after intravenous glucagon administration has been noted by Shoemaker and Van Itallie (3).

[4] Glucagon, crystalline, "insulin free" was generously supplied by Eli Lilly Company.

[5] Synthetic l-epinephrine—Winthrop-Stearn.

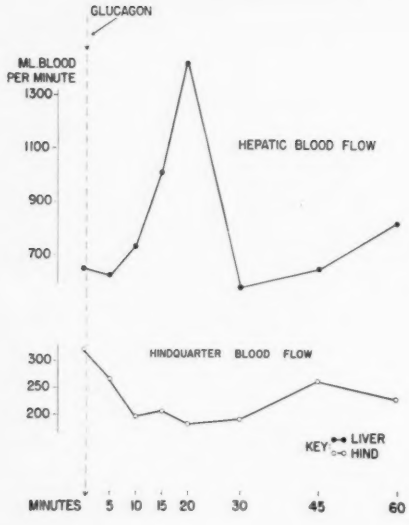

FIG. 1. Effect of glucagon on the mean hepatic blood flow of 5 dogs and the mean hindquarter blood flow of 5 normal, conscious dogs.

In the present studies hepatic glucose production rose rapidly during the first 20 minutes, and fell to control levels 40 to 45 minutes after glucagon (Fig. 2). Changes in hindquarter and nonhepatic splanchnic area concentration gradients indicated an increase in glucose uptake which was apparent sometimes within 2 minutes. The time relationship of the hindquarter glucose metabolic changes coincided with the changes in hepatic glucose metabolism. Femoral venous and arterial blood levels of glucose rose rapidly but remained elevated for as long as 90 minutes.

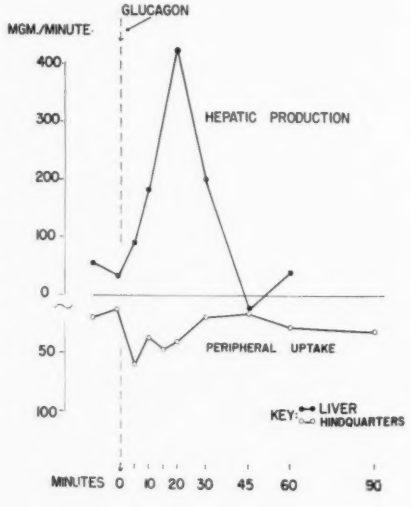

FIG. 2. Effect of glucagon on hepatic glucose output and hindquarter glucose uptake.

Mean changes of five dogs studied in the hepatic series and six dogs in the hindquarter series. A negative arterio-venous concentration gradient multiplied by the rate of blood flow is shown as a milligram per minute output, or production. A positive arterio-venous concentration gradient multiplied by the rate of blood flow is shown as a milligram per minute uptake.

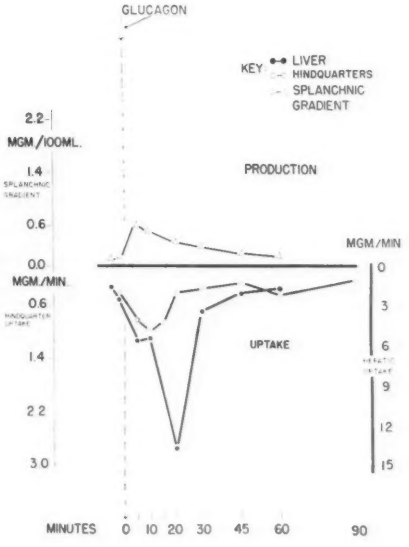

FIG. 3. Effect of glucagon on pyruvate metabolism by the liver, nonhepatic splanchnic area and the hindquarters.

The "splanchnic gradient" expressed in milligrams per 100 ml. of blood represents the arterio-venous concentration gradient between the splenic artery and the portal vein. A negative value for this gradient is shown as a production while a positive value is shown as an uptake. For further details, see legend for Figure 2.

Pyruvate Changes

Femoral venous and arterial blood levels of pyruvate rose during the first 15 minutes after glucagon but then decreased to or below control values. These changes accompanied an early increase in the nonhepatic splanchnic area output of pyruvate and a marked increase in both hepatic and hindquarter pyruvate utilization (Fig. 3).

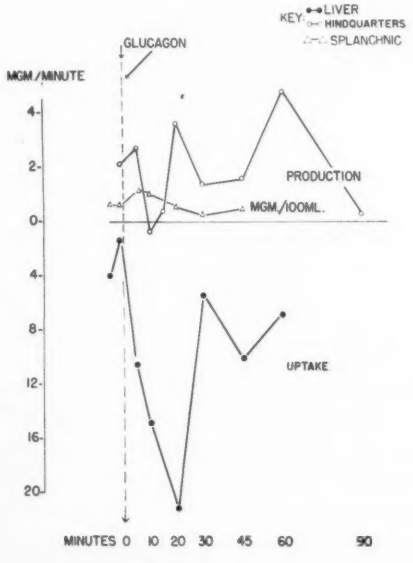

FIG. 4. Effect of glucagon on lactate metabolism. For details see legends for Figures 2 and 3.

Lactate Changes

Femoral venous and arterial blood levels of lactate showed an initial rise 15 minutes after glucagon; the rise was minimal and of short duration. Hindquarter production of lactate increased and concentration gradients across the nonhepatic splanchnic area indicated an increased output (Fig. 4). Fluctuations in the rate of metabolism across the hindquarters in the control period suggested that small degrees of both production and utilization were occurring from minute to minute although the average total effect was one of production (Fig. 4). These fluctuations were present in each animal as well as in the group as a whole.

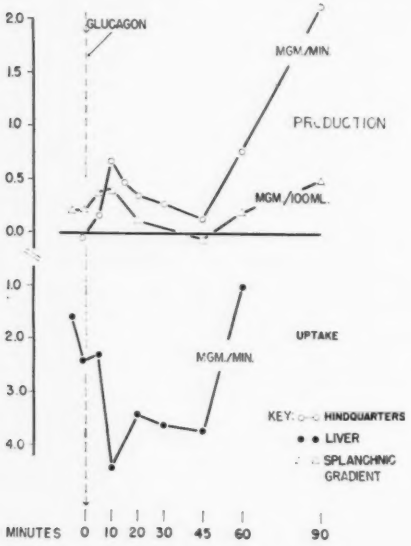

FIG. 5. Effect of glucagon on citrate metabolism. For details see legends for Figures 2 and 3.

Citrate Changes

Femoral venous and arterial blood levels of citrate decreased 15 minutes after glucagon despite the fact that the rate of hind limb production and nonhepatic splanchnic area output increased (Fig. 5). The decrease in blood concentrations reflected the marked increase in hepatic citrate uptake (Fig. 5).

Effects of Glucagon Administered to Fasted Dogs

Glucagon administered to three dogs after a 3 to 5 day fast failed to increase hepatic glucose production in one and produced only a minimal increase in the remaining two animals (Fig. 6). Elevations in peripheral glucose levels were insignificant in two and minimal in the third. Hindquarter uptake of glucose in this third animal increased but to a lesser degree and for a shorter period of time than that observed in normal dogs

(cf. Figs. 2 and 6). Nonhepatic splanchnic area uptake of glucose was increased but to a lesser degree than that observed in normal dogs. Although the effects of glucagon on glucose metabolism were less pronounced after fasting, the effects on pyruvate, lactate and citrate metabolism were not. Resting hepatic uptake of citrate in the fasted animals was 6.5 mg./min. in contrast to a rate of 1.5 mg./min. in the normal animals of comparable weight. When glucagon was administered to the fasted animals hepatic uptake of citrate increased to 14 mg./min. (Fig. 6). The increase in hindquarter uptake of pyruvate following glucagon was somewhat delayed in

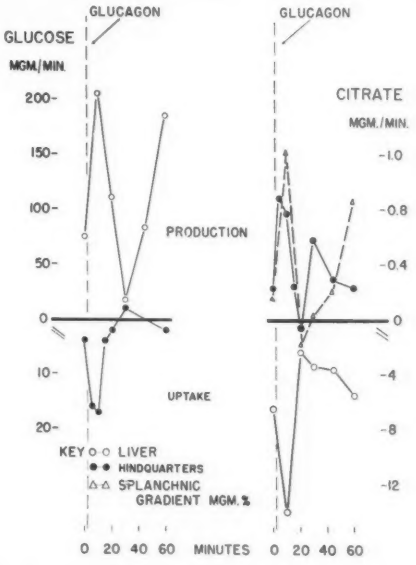

FIG. 6. Effect of glucagon on glucose and citrate metabolism in three starved dogs.

For details see legends for Figures 2 and 3.

onset but was not decreased in degree by starvation (Fig. 7). Lactate metabolism across both the liver and peripheral areas was not significantly different between the normal and the starved dogs of this series (Fig. 7).

Effects of Administered Epinephrine in Normal Dogs

The administration of epinephrine in doses of 1 and 5 μg/kg. changed the arterio-venous glucose concentration gradient across the nonhepatic splanchnic area from negative to positive indicating a change from an uptake to an output. This was in striking contrast to the uptake observed at rest and after glucagon administration observed in over 100 determinations on 17 dogs. Although increases and decreases in gradients might merely reflect changes in blood flow, a change from a positive to a negative gradient cannot be caused by a change in blood flow. A definite increase in the pyruvate, lactate and citrate gradient was also observed across the nonhepatic splanchnic area.

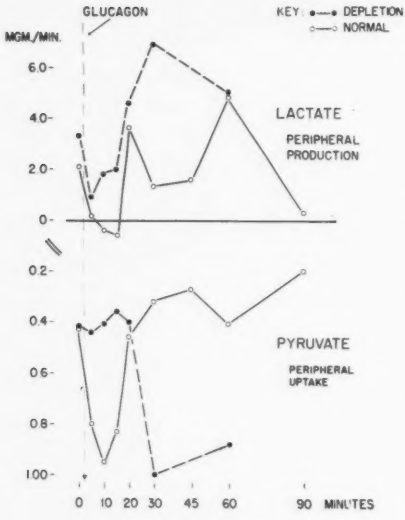

FIG. 7. Effect of glucagon on hindquarter pyruvate and lactate metabolism in five normal and three starved dogs.

Solid circles = starved dogs; open circles = normal animals. For further details see legend for Figure 2.

Epinephrine (1.0 μg/kg., i.v.) increased hepatic glucose production three-fold and pyruvate and lactate uptake almost six-fold (Fig. 8). Levels had returned to control within fifteen minutes even though nonhepatic splanchnic area output and circulating blood levels occasionally remained above control values for 90 minutes.

DISCUSSION

The results of experiments with glucagon are consistent with the observations of others (10, 11) and suggest that glucagon has a dual coordinated

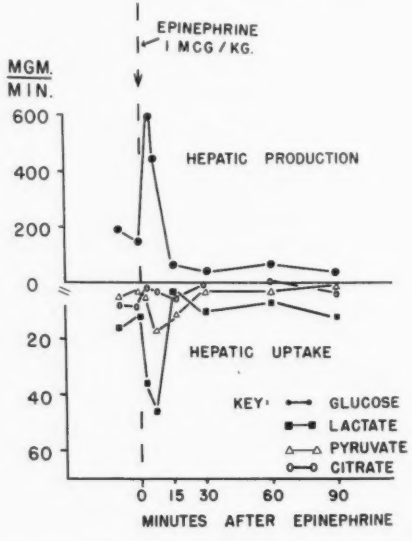

FIG. 8. Effect of epinephrine on hepatic metabolism of glucose, lactate, pyruvate and citrate in three normal, conscious dogs.

For details see legend of Figure 2.

effect on the release of hepatic glucose and an increase of peripheral glucose utilization. The latter effect, manifest as an increase in glucose uptake despite a decrease in blood flow, may be the result of a direct action of glucagon or may be a consequence of the increased hepatic glucose output and the subsequent rise in blood glucose levels. The data of the present study indicate that glucagon has an integrated effect on both hepatic and peripheral metabolism of pyruvate, lactate and citrate as well as glucose. At rest there exists a homeostatic balance between the hepatic uptake of lactate and citrate and the net peripheral production of these metabolites; glucagon increases the rates of normal resting lactate and citrate metabolism but maintains the same inverse relation between the liver and periphery. Glucagon also simultaneously increases resting hepatic and hindquarter pyruvate utilization. Fasting was found to depress the effect of glucagon on hepatic and peripheral glucose metabolism but it did not depress the effects of glucagon on pyruvate, lactate or citrate metabolism. The rather pronounced effects of glucagon on lactate, pyruvate and citrate which occurred without hyperglycemia in the fasting state also suggest a direct peripheral action of glucagon. These effects, therefore, are probably not mediated by the release of endogenous insulin or by an increased peripheral uptake of glucose.

Other effects of glucagon, similarly independent of its hyperglycemic effect, have been suggested by others. These include the release of free fatty acids from adipose tissue and the effects on renal function (12, 13). Changes in the renal clearance of sodium and potassium such as Elrick observed after glucagon are known to effect citrate excretion. The effect of glucagon on citrate excretion remains to be studied.

The kidney is believed to be the major organ utilizing citrate (9) and the bone has been described as the major, if not the only, source of endogenous citrate (14). Nevertheless we observed a significant uptake of citrate across the liver at rest which was comparable to that observed across the kidney and which increased 3- to 6-fold following glucagon administration. We also observed a constant output of citrate across the nonhepatic splanchnic area which includes no bone.

Peripheral metabolism of pyruvate varied from one area to another. These differences may to some extent reflect metabolic differences between the several tissues included in the regions—e.g., the presence of bone in the hindquarters and the absence of both bone and skeletal muscle in the nonhepatic splanchnic area. The present study also indicates considerable recycling of glucose and organic acids between the liver and the peripheral areas; moreover, it suggests an interrelationship between one peripheral area and another.

Both glucagon and epinephrine have been shown to increase hepatic glycogenolysis. Their effect on circulating blood concentrations of pyruvate, lactate and citrate are not the same, however (15, 16). The differences have generally been assumed to reflect the increase in muscle glyco-

genolysis and decrease in glucose oxidation produced by epinephrine (17, 18). The present studies show that, in general, the effect of epinephrine was similar to that of glucagon except that we failed to find an increased hepatic uptake of citrate or hindquarter uptake of pyruvate after epinephrine administration. One might speculate that these differences explain the increase in circulating blood levels of these substances following the administration of epinephrine. However, until more complete examination of all metabolic regions and tissues have been made in the intact animal such a conclusion would be premature.

Drury and Wick (19) observed that C^{14} lactate was more readily oxidized in rabbits than was C^{14} glucose and that very little labeled lactate was converted to glucose or glycogen. These findings appear somewhat at variance with those reported herein. However, the experimental conditions of Drury and Wick were not comparable to the present study in which an increased hepatic uptake of pyruvate and lactate was measured directly during the first fifteen minutes after epinephrine. Drury and Wick measured the appearance of the labeled lactate into glycogen over a period beginning 15 to 20 minutes after epinephrine administration. This period might well represent a period of compensatory changes, rather than the period of direct epinephrine action.

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INCORPORATION OF ACETATE-1-C^{14} INTO TESTOSTERONE AND 3β-HYDROXYSTEROLS BY THE CANINE TESTIS[1]

NORMAN R. MASON[2] AND LEO T. SAMUELS

Department of Biological Chemistry, University of Utah College of Medicine, Salt Lake City, Utah

ABSTRACT

Acetate-1-C^{14} was infused into the spermatic arteries of dogs at a constant rate before and after human chorionic gonadotropin was injected into the spermatic circulation to one testis. The blood from the spermatic vein was collected and the amounts and specific radioactivities of testosterone in successive samples were determined. In several experiments gonadotropin-stimulated and unstimulated testes were removed at the end of the collection, the various particulate fractions separated by high speed centrifugation and the specific activity of the digitonin-precipitable material in each determined.

The specific activity of the testosterone rose very soon after the introduction of the gonadotropin. In most cases the output of testosterone also increased, but even in those cases where this did not occur the specific activity was higher. The pool of intermediates between acetate and testosterone, therefore, seemed to be quite small in the actively secreting testis and the point of action of human chorionic gonadotropin appeared to be before the rate limiting step in testosterone biosynthesis.

There was no correlation between the specific radioactivity of the various digitonin-precipitable fractions and that of testosterone before and after gonadotropin. The specific activity of the testosterone after the gonadotropin injection was always higher than any digitonin precipitable fraction, and in most cases this was true before gonadotropin injection. If cholesterol is an obligatory intermediate in the biosynthesis of testosterone in the testis, the pool involved is only a minor portion of any of the fractions separated.

BRINCK-JOHNSEN and Eik-Nes (1) and Hollander and Hollander (2) have both shown that human chorionic gonadotropin (HCG) has an immediate effect on the production of the two androgens, testosterone and 4-androstene-3,17-dione, shown by West *et al.* (3) to be the secretory products of the canine testis. Earlier, Brady (4) reported that the incorporation of acetate into testosterone by dog testis slices was greater when HCG was included in the incubation medium. While the radioactivity from acetate-1-C^{14} in the testosterone fraction was increased about tenfold,

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[2] Investigator, Howard Hughes Medical Institute. Present address: The Endocrine Laboratory, Department of Medicine, University of Miami School of Medicine, Miami, Florida.

that in cholesterol was not significantly affected. He could not determine, however, whether the increased activity in the testosterone zone was due to greater amounts of steroid of the same specific activity or whether there was actually increased specific activity.

Because HCG had such a marked effect on acetate incorporation into testosterone but no measurable influence on incorporation into cholesterol, Brady raised the question of whether cholesterol was an essential precursor of the androgen. As a possible alternative, Hechter and Pincus (5) have proposed a scheme by which an unknown compound "X" synthesized from acetate may either be converted into cholesterol or directly into the steroid hormones.

In this investigation acetate-1-C^{14} was infused into the arterial circulation of the testes of anesthetized dogs and its incorporation into testosterone was determined in the spermatic venous blood. This was compared with the incorporation into the digitonin-precipitable sterols of testes from the same animal before and after injection of HCG. It was found that HCG consistently increased the incorporation into testosterone but not into testicular 3β-hydroxysterols.

METHODS

Male mongrel dogs weighing 18 kg. or more were obtained from the local pound and in most instances injected intramuscularly with 500–700 I.U. of HCG[3] distributed over periods of 4–8 days. The animals were anesthetized by injecting 32.5 mg. sodium pentobarbital per kg. intravenously, a longitudinal incision was made on each side of the midline in the caudad portion of the abdomen, and the spermatic artery and veins of each testicle were exposed below the *ligamentum inguinale*. The femoral artery in each thigh was also exposed, and the animal was then given 50 mg. of heparin in saline solution intravenously. Each femoral artery was connected to the spermatic artery on the same side by means of a tapering polyethylene cannula. A needle connected to a syringe type infusion pump by a polyethylene tube was inserted into the wall of the arterial cannula, and the infusion of acetate-1-C^{14} of specific activity 1 mC per mmole was begun. The left spermatic vein was then cannulated (1). In one experiment both spermatic veins were cannulated.

Spermatic venous blood was collected for 10 to 20 minute intervals. Each sample represented 40–70 ml. of whole blood; the volume was replaced by infusion of isotonic saline or heparinized dog blood. After a control period of acetate infusion, 500–1000 I.U. of HCG were injected into the left arterial cannula and collection of blood continued for one to three hours thereafter. At the end of the experiment the animal was sacrificed and the testes were removed for cell fractionation and cholesterol analysis.

The blood samples were centrifuged and the plasma extracted four times with equal volumes of freshly distilled chloroform. The chloroform extracts were evaporated to dryness, the residue taken up in 70% methanol, and fat and cholesterol precipitated at −10° C (6). After centrifuging at 3000 R.P.M. for 30 min. in an International Refrigerated Centrifuge, the supernatant fluid was decanted, evaporated to dryness under nitrogen, and the residue chromatographed in the hexane-benzene:formamide system of Zaffaroni (7). The chromatogram was developed for three hours and then dried overnight at room temperature in moving air.

[3] A.P.L. chorionic gonadotropin was generously supplied by Ayerst Labs.

The radioactivity in the areas on the strip corresponding to testosterone and androstenedione was determined by the method of Berliner *et al.* (8). Quantitative estimation of the steroids was done by scanning the strips in the Cary double beam spectrophotometer with the wave length set at 240 mμ (9). Receoveries of testosterone by this method averaged 78% with a range of 70–100%. The error in the determination of specific activities was ±12%.

As soon as the testes were removed, they were weighed, minced and homogenized. Fractionation of a 10% tissue suspension in 0.25 molar sucrose was carried out in a Spinco Model L ultracentrifuge according to the procedure of Bucher and McGarrahan (10) except that the centrifugation at 60,000×g. was eliminated.

The various cellular fractions were saponified with 10% KOH in 50% ethanol at 50° C for 15 hours. Each solution was then evaporated under nitrogen to 0.5 volume, diluted with an equal volume of water, and extracted four times with twice the volume of hexane. The extracts were washed with water, evaporated to dryness under nitrogen and the residue taken up in 1 ml. of ethanol. To this solution 0.5 ml. of water and 1 ml. of a 0.5% solution of digitonin in 50% ethanol were added and the digitonide allowed to precipitate for three hours at room temperature. The mother liquor was decanted after centrifuging and the digitonide precipitate washed with 2 to 3 ml. of 4:1 acetone-chloroform (v/v). The digitonide was dissolved in 1 ml. of methanol, and one aliquot was plated for determination of radioactivity while another was used for quantitative estimation of cholesterol according to the Zak colorimetric method (11).[4] The recovery of radioactive cholesterol added to tissue fractions averaged 75% by this method and the error in the determination of specific activity was ±7%.

RESULTS

The effects of previous intramuscular injection of HCG on blood flow through the testes and on testosterone secretion are illustrated in Table 1. A minimum of about 70 I.U. of HCG per 10 kg. body weight per day injected over a period of four days or more was required to produce consistently measurable testosterone concentrations in spermatic venous blood before intra-arterial injection of HCG. In such experiments (No. 10–No. 14) the blood flow also was higher than in the first eight experiments in which 60 I.U. per 10 kg. or less per day were injected. Since intra-arterial injection of HCG increased the output of testosterone promptly in most of the experiments without affecting blood flow, the effects of the pre-injections on the vascular system cannot be the primary mechanism by which testosterone output is increased.

The most consistent stimulation of testosterone secretion by the intra-arterial injections of HCG appeared to be associated with intermediate levels of HCG during the preparative period. If the interstitial cells were not sufficiently stimulated the effect, while measurable, was not as great; on the other hand, if the stimulation had been too great a further acute increase in testosterone output could not be consistently elicited.

In all the experiments where the specific activity of testosterone could be determined both before and after the injection of HCG, the gonadotropin

[4] We are indebted to Dr. Ralph Elefsen, Section of Biochemistry, Mayo Clinic, Rochester, Minnesota, for information pertaining to the Zak method as used for tissue cholesterol analysis.

TABLE 1. BLOOD FLOW AND TESTOSTERONE CONCENTRATION OF DOG SPERMATIC VEIN BLOOD BEFORE AND AFTER HCG INTRA-ARTERIALLY

Experiment	HCG preinjection dose (I.U./10 kg./day)	Period of preinjection (days)	Collection[1] period (min.)	HCG intra-arterially (I.U./10 kg.)	Average blood flow (ml./10 kg./min.)	Ave. testosterone production (μg/10 kg./min.)
1	0	0	195 min. before		0.35	0.03
			150 min. after	53	0.39	0.03
2	0	0	175 min. before		1.0	0.01
			110 min. after	192	0.80	0.21
3	0	0	255 min. before		0.73	0.00
			75 min. after	278	0.68	0.16
4	39	15	180 min. before		0.98	0.00
			60 min. after	24	0.68	0.16
5	39	16	40 min. before		1.0	0.00
			195 min. after	834	0.86	0.70
6	57	14	230 min. before		1.1	0.04
			125 min. after	80	0.86	0.71
7	60	12	95 min. before		0.64	0.10
			175 min. after	690	0.63	0.30
8	62	14	80 min. before		2.5	0.00
			80 min. after	435	2.0	0.20
9	74	9	120 min. before		1.74	0.08
			45 min. after	740	0.95	0.30
10	78	10	120 min. before		1.1	0.24
			120 min. after	435	1.3	0.43
11	80	11	60 min. before		2.0	0.37
			90 min. after	435	2.5	0.83
12	114	8	20 min. before, right		2.7	0.53
			80 min. before, left	217	2.1	0.58
			80 min. after, right		1.4	0.37
			80 min. after, left/(HCG)	217	1.8	0.36
13	121	10	87.5 min. before		2.0	0.10
			37.5 min. after	151	2.5	0.55
14	131	8	50 min. before		2.4	0.52
			50 min. after	500	1.9	0.47
15	460	6	30 min. before		1.2	2.0
			165 min. after	690	1.7	2.2

[1] Collection periods refer to times of collection *before* and *after* intra-arterial injection of HCG.

caused a significant increase. This occurred whether or not the absolute output of the hormone was increased (Table 2). If the preinjections of HCG were less than 55 I.U. per kg. per day, however, no measurable incorporation of acetate carbon could be detected, before or after the intra-arterial HCG administration. The increase in specific activity was not simply due to the coincidence of the HCG injection and the time required to use up intermediates in testosterone biosynthesis because there was no correlation

TABLE 2. CONVERSION OF ACETATE TO TESTOSTERONE IN DOG TESTES BEFORE AND AFTER THE INTRA-ARTERIAL INJECTION OF HCG

Experiment no.:	7	9	10	11	12	13	14
HCG Preinjections, I.U./10 kg./day	60	74	78	80	114	121	131
Ave. acetate infusion, μc/min.	0.20	0.38	0.35	0.50	0.30	0.22	0.17
HCG, Intra-arterial Inj., I.U./10 kg.	690	740	435	435	217	151	500
Time blood collected:*							
Before HCG, min.	95	120	120	60	80	88	50
After HCG, min.	200	45	120	90	80	38	50
Testosterone spec. act., Ave.:							
Before HCG, cpm/mg. $\times 10^{-2}$	308	2480	1145	1630	848	80	105
After HCG, cpm/mg. $\times 10^{-2}$	550	7500	2130	1315	1720	336	190
Testosterone produced, Ave.:							
Before HCG, mμmoles/min.	1.01	0.75	1.92	2.95	4.63	1.15	3.62
After HCG, mμmoles/min.	3.02	2.81	3.43	6.63	2.88	6.30	3.26
Acetate conversion, Ave.:							
Before HCG, % of infused	.002	.006	.008	.013	.017	.001	.003
After HCG, % of infused	.011	.087	.027	.023	.022	.013	.005

* The blood was collected in 10–30 minute samples. Testosterone and testosterone specific activity were determined on each sample. The values given are averages of all samples before or after HCG.

between time or total testosterone output before HCG injection and the increase in specific activity after injection. Further, in experiment 12 blood was collected simultaneously from both the left and the right testicular veins while HCG was given only to the left testis. In spite of the fact that the total testosterone secretion after the HCG injection was not significantly increased, the specific activity of testosterone secreted by the testis receiving HCG increased both in relation to the specific activity of testosterone secreted before HCG and in relation to the specific activity of testosterone secreted by the testis not receiving HCG. In the uninjected testis the specific activity did not change significantly over the same period of time (Table 3). The effect on specific radioactivity, therefore, appears to be directly related to the action of HCG.

TABLE 3. TESTOSTERONE SPECIFIC ACTIVITIES IN EXPERIMENT 12

Acetate was infused into, and blood was collected from, both testes but HCG was injected into the circulation of the left testis only

Collection period min.	Testosterone specific activity cpm/mg. $\times 10^{-2}$	
	Left	Right
20	812	—
20	725	—
20	993	—
20	873	1340
	Inj. HCG 500 I.U.*	
20	1570	923
20	1830	968
40	1450	1110

* The HCG injection was made into the arterial supply of this testis.

TABLE 4. TESTOSTERONE-ANDROSTENEDIONE RATIOS BEFORE AND AFTER INTRA-ARTERIAL ADMINISTRATION OF HCG

Experiment no.	Ratio of testosterone to androstenedione		Average specific activity			
			Testosterone		Androstenedione	
	Before HCG	After HCG	Before HCG cpm/mg. $\times 10^{-2}$	After HCG cpm/mg. $\times 10^{-2}$	Before HCG cpm/mg. $\times 10^{-2}$	After HCG cpm/mg. $\times 10^{-2}$
6	2.6	2.7	—	8.1	—	8.3
7	4.8	3.6	90	250	70	350
9	1.9	2.0	790	4110	880	1970
13	3.7	3.1	520	970	690	1020

In four experiments the specific activity of 4-androstene-3,17-dione could also be determined in spermatic vein blood before and after the intra-arterial injection of HCG. The specific activity of this compound as summarized in Table 4 was comparable to that of the accompanying testosterone, and the testosterone-androstenedione ratio did not change significantly.

The identities of both testosterone and androstenedione were established by chromatography of derivatives of individual samples and by recrystallization of a pooled sample of the unknown diluted with the appropriate carrier material. The material corresponding to testosterone on the paper chromatograms was treated with acetic anhydride and pyridine, 1:2 v/v, overnight at room temperature. Chromatography of the resulting material indicated that an acetate had been formed corresponding to testosterone acetate and without a change in specific activity. The unknown was also treated with chromium trioxide in glacial acetic acid, and a compound was formed which corresponded to androstenedione on chromatography. Similar procedures were carried out with the unknown material corresponding to androstenedione on the chromatograms. In this case, no change in the chromatographic behavior of the original material was noted after acetylation, and the specific activity remained unchanged. When the pooled samples corresponding to testosterone and androstenedione were recrystallized with 10 mg. of the appropriate unlabelled steroid, there was no significant change in specific activity, either on the first crystallization or after recrystallization from different solvents (Table 5).

TABLE 5. RECRYSTALLIZATION OF TESTOSTERONE AND ANDROSTENEDIONE TO CONSTANT SPECIFIC ACTIVITY

	Testosterone		Androstenedione	
	Solvent	Specific activity cpm/mg. $\times 10^{-3}$	Solvent	Specific activity cpm/mg. $\times 10^{-3}$
Original	—	184	—	113
First recrystallization	Acetone	183	Ethanol	132
Second recrystallization	Benzene	193	Methanol	110
Third recrystallization	Methanol	182	Methanol	116

TABLE 6. SUBCELLULAR DISTRIBUTION AND SPECIFIC RADIOACTIVITY OF DIGITONIN PRECIPITABLE STEROLS

Expt.	Subcellular fraction[1]	Digitonin precipitable sterols				Sp. act. of plasma testosterone	
		Unstimulated testis		Stimulated testis			
		Amt.[2] μmoles	Sp. act. cpm/mg.	Amt.[2] μmoles	Sp. act. cpm/μmole	Unstim. cpm/mg.	Stim. cpm/mg.
12	Mitochondria	1.3	7450	2.5	7270	84800	172000
	L. Microsomes	3.5	8440	2.7	8640		
	S. Microsomes	3.5	14540	3.3	9250		
	Supernatant	1.0	12100	0.8	10680		
13	Mitochondria	1.9	6480	1.2	4000	8000	33600
	L. Microsomes	4.8	5060	5.3	3900		
	S. Microsomes	5.2	8200	6.6	4320		
	Supernatant	4.3	10050	2.9	9310		
14	Mitochondria	11.6	4920	7.0	3220	10500	19000
	L. Microsomes	3.3	3710	6.6	2980		
	S. Microsomes	2.3	3620	3.7	2290		
	Supernatant	1.2	6020	3.1	3080		

[1] For methods of centrifugal separation see text.
[2] Estimated by colorimetric method of Zak (11).

In a number of experiments the specific activities of the digitonin precipitable material isolated from mitochondrial, microsomal and cytoplasmic fractions of the HCG stimulated testes were compared with those of similar fractions from the corresponding unstimulated testis. In none of the fractions did the gonadotropin produce a consistent change. In Table 6 the values for the mitochondrial, microsomal and supernatant fractions are compared with the specific activities of the spermatic vein testosterone in the three experiments where all values were obtained. Not only is there no consistent change in the specific activities of the digitonin precipitable material with stimulation, but there is no relationship to the specific activities of the testosterone. Moreover, the specific activities of the testosterone after HCG injection are higher than those of any of the corresponding digitonin fractions.

TABLE 7. PURIFICATION OF CHOLESTEROL AS THE DIGITONIDE AND AS THE DIBROMIDE[1]

	Cholesterol digitonide, sp. act. as cholesterol	Cholesterol dibromide, sp. act. as cholesterol
	cpm/mg.	cpm/mg.
Experiment 11		
Original	363	127
Twice recrystallized	375	161
Experiment 13		
Original	357	190
Twice recrystallized	374	178

[1] 20 mg. pure cholesterol were added to the combined sterol fractions from the different cellular components separated by centrifugation from a testis injected intra-arterially with HCG. Precipitation with digitonin and purification through the dibromide were then carried out.

The amounts of labelled digitonin precipitable material in the individual samples were too small to yield significant specific activities after the necessary dilution to permit purification through the dibromide, but in one experiment the fractions from the various cellular fractions were combined and carried through the procedure as described by Schwenk and Werthessen (12). As these authors found with digitonin precipitable material in the liver, the specific activity of the regenerated cholesterol was only half that of the fraction recrystallized with cholesterol before the purification (Table 7). Thus, acetate carbons had apparently been rapidly incorporated into digitonin precipitable material other than cholesterol. This makes the over-all difference between the specific activities of cholesterol and testosterone even greater and raises the question of the unknown intermediates as more direct precursors of the hormone.

DISCUSSION

Two important characteristics of gonadotropic action on testosterone biosynthesis have been established by these experiments. First, since testosterone specific activities were approximately doubled by HCG injection in animals where the steroid output, already high, was not increased, the effect of the gonadotropin must be on some step (or steps) in biosynthesis of testosterone which precedes the rate limiting step. Second, since a marked increase in rate of acetate incorporation into testosterone follows by a very short interval the introduction of HCG into the spermatic artery, the pool of intermediates must be relatively small.

A series of enzymic reactions involved in the formation of testosterone from the 21-carbon steroid 5-pregnene-3β-ol-20-one by the testis have been rather clearly delineated. They involve a conversion of this compound to progesterone under the influence of a 3β-hydroxysteroid dehydrogenase (13) and an isomerase (14), the conversion of progesterone to 17α-hydroxyprogesterone and the subsequent oxidative splitting of the side chain (15) with the release of acetic acid (16) to form 4-androstene-3,17-dione, and the reduction of the latter reversibly to testosterone through the action of a 17β-hydroxysteroid dehydrogenase. Of these, the only compounds present in bovine testis extracts in more than trace amounts are 5-pregnen-3β-ol-20-one (17) and testosterone (18). Thus in the bovine a limiting reaction would seem to be the dehydrogenation of pregnenolone. Since the sequence seems to be the same in other species this may also be a rate limiting step in the dog. Since the increase in specific activity without a rise in total output of testosterone in some cases would mean that HCG acts before a rate limiting step, the gonadotropic effect would seem to be before pregnenolone.

In view of the low, usually unmeasurable, concentrations of all these compounds in the testes it is not surprising that carbon from circulating acetate would appear quickly in the venous testosterone during active

testosterone biosynthesis and secretion, providing a large cholesterol pool was not involved. This seems to be the case since, as Brady (4) observed, a marked increase in acetate incorporation in testosterone after HCG is not associated with any change in incorporation into total cholesterol. Moreover, our results show that the increase is in specific activity as well as total incorporation. The limited nature of the cholesterol pool, if it exists, is also indicated by the lack of influence of HCG injection on the specific activities of the digitonin-precipitable material in the separate particulate fractions. This is emphasized even more by the fall in specific activity when the digitonin-precipitable material which was recrystallized to constant specific activity with cholesterol was subjected to the purification through the dibromide. The evidence presented here does not strengthen the hypothesis of cholesterol as an obligatory intermediate. On the other hand, the experiments of Loud and Bucher (19) showing that only about 1% of the small hepatic squalene pool is actually involved in cholesterol biosynthesis make any conclusions uncertain until similar studies are carried out with the cholesterol-testosterone sequence in the testis or an alternate intermediate is demonstrated.

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ADRENO-CORTICAL AND MEDULLARY RESPONSES TO ADENOSINE 3′,5′-MONOPHOSPHATE[1]

JAMES G. HILTON, OSCAR R. KRUESI, RELJA I. NEDELJKOVIC AND LOUIS F. SCIAN[2]

Department of Medicine, St. Luke's Hospital, New York, N. Y.

ABSTRACT

By means of direct arterial perfusion of the isolated intact dog adrenal glands it has been found that adenosine 3′,5′-monophosphate stimulates the continuous secretion of hydrocortisone. During the period of administration of the nucleotide, the character and magnitude of steroid secretion was comparable to that seen during perfusion of ACTH. Steroidogenesis by rat and guinea pig adrenal slices incubated with 3′,5′-monophosphate was also studied, and a species difference in sensitivity of response has been demonstrated.

Cathechol amine secretion, studied in the dog adrenal preparation, was unaffected by perfusion of either ACTH or the nucleotide.

IT HAS been shown that adrenocorticotropic hormone will increase the phosphorylase activity of steer adrenal cortical slices (1) and extracts (2) *in vitro*. This ACTH activation of adrenal phosphorylase is mediated through adenosine 3′,5′-monophosphate (3′,5′ AMP) (3). Furthermore, 3′,5′ AMP added to rat adrenal gland slices incubated *in vitro* elicits a response in corticosteroid production equal to or greater than that produced by ACTH (4).

Since *in vitro* systems have limited physiologic potentials and since it has been shown that phosphorylase activity *in vivo* is unidirectional as contrasted to a reversible behavior *in vitro* (5), we thought it germane to study the effect of 3′,5′ AMP on the isolated perfused intact dog adrenal gland preparation.

In addition, further studies were performed on adrenal slice incubates of the guinea pig since guinea pigs, as do dog and man, produce hydrocortisone (cpd F) as the major adrenal steroid (6). These studies were compared with results obtained from the incubation of rat adrenal slices since the rat, as well as the steer (7), produces corticosterone (cpd B) as the major adrenal steroid.

The data to be reported show that both isolated perfused dog adrenal glands and guinea pig adrenal slices respond to 3′,5′ AMP with an increase in hydrocortisone secretion, whereas rat adrenal slices produce

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[2] Fellow of The New York Heart Association.

corticosterone after stimulation by the nucleotide. Furthermore, it was noted that catechol amine output from the isolated perfused dog adrenal gland is not increased by either ACTH or 3′,5′ AMP.

EXPERIMENTAL

Material—Adrenal glands, freshly obtained from fifteen Hartley line guinea pigs of both sexes (mean weight 299 gm.; range 267 to 338 gm.) and twenty-eight male Sprague-Dawley rats (mean weight 198 gm.; range 174 to 220 gm.) were used in the *in vitro* studies.

The experiments, designed to investigate the direct effect of 3′,5′ AMP on the adrenal glands *in vivo*, utilized six pairs of adult dogs, *i.e.* a donor and a recipient in each experiment. The *in situ* perfusion of the recipient dog adrenals (the arterial inflow as well as venous outflow being completely isolated from the systemic circulation), with heparinized arterial blood of the donor animal was carried out by means of a pump according to the method described by Hilton *et al.* (8). The 3′,5′ AMP sample, Lot No. 128-679-2 or Lot No. 79651, purchased from Sigma Chemical Company, and Lot No. AOC6001 kindly donated by the Schwartz Biochemical Company, was added to the arterial circuit leading to the adrenal preparation. The ACTH sample used in the same manner at the end of each perfusion experiment was a product of the Upjohn Company (Corticotropin Injection). The same substances were used in the *in vitro* study of steroidogenesis by guinea pig and rat adrenals.

All operations were performed under sodium pentobarbital anesthesia, induced intraperitoneally in guinea pigs and rats (50 mg./kg. of body weight) and intravenously in dogs (26 mg./kg. of body weight). In three instances donor and recipient dogs were hypophysectomized, as described by McClean (9), the day before the experiment.

Incubation of slices—Adrenals, removed from 28 rats and 15 guinea pigs, were cut into three equal parts. Both rat and guinea pig adrenal thirds were then divided into three groups and incubated in oxygenated Krebs-Ringer-bicarbonate-0.01 M glucose medium as described by Haynes *et al.* (1, 4). After incubation the medium was removed, the slices rinsed with 1.5 to 2.0 ml. of fresh incubation solution and then the medium, with added rinsings, extracted three times with three separate aliquots of purified methylene chloride. The pooled methylene chloride extracts were evaporated to dryness under vacuum at a water bath temperature of 40° C before chromatography.

Blood samples—The concentration of hydrocortisone both in the arterial plasma entering the recipient's adrenal glands as well as in venous plasma leaving the glands was determined by the method of Peterson *et al.* (10). Epinephrine and norepinephrine determinations were done by the method of Weil-Malherbe and Bone (11). Adrenal plasma flow was calculated from the measured adrenal venous blood flow and the hematocrit of the adrenal venous blood. The rate of hydrocortisone and catechol amine secretion by the adrenal glands was calculated by multiplying the arteriovenous concentration difference by the adrenal plasma flow and is expressed in μg/minute.

Descending paper chromatography—Chromatographic fractionation of steroids from the adrenal gland incubates was done by a modified Bush system (toluene—methanol—water, 10:7:3) (12). The method of Peterson (13), (cyclohexane—benzene—methanol—water, 2:2:2:1), was used for chromatography of the dog plasma extracts. The chromatographic mobility of unknown steroids was always compared with that of reference standards of hydrocortisone and corticosterone containing 2, 5, and 10 μg of authentic steroid (free alcohol) run under identical conditions. After staining with blue tetrazolium, the developed chromatograms were analyzed in a recording and integration densitometer (Analytrol, Spinco, Model RB) equipped with 550 mμ interference filters and the B-2 cam for colorimetry according to Kittinger (14). Unknowns were calculated from stand-

TABLE 1. EFFECT OF 3′,5′ AMP ON HYDROCORTISONE (F) SECRETION FROM ISOLATED PERFUSED DOG ADRENAL GLANDS

Following control periods, 3′,5′ AMP was infused at doses and duration indicated and the increment rise in cpd F secretion (ΔF) in the adrenal venous effluent over the control secretion rate is shown and compared with similar ΔF secretion following injection of ACTH at the end of each experiment.

Expt. no.	Control F μg/min.	Adenosine 3′,5′-monophosphate			After ACTH[1]	AMP[2]
		Dose	Duration	ΔF μg/min.	ΔF μg/min.	responsiveness
		Hypophysectomized				
1	0.1	0.1 mg./min.	42 min.	3.2	5.8	55%
2	0.2	0.1 mg./min.	24 min.	2.3	2.1	110%
3	0.1	0.1 mg./min.	25 min.	2.6	1.9	136%
		Non-hypophysectomized				
4	4.3	0.1 mg./min.	15 min.	5.6	2.8	200%
5	2.0	1.0 mg./min.	10 min.	4.0	2.8	143%
6	4.3	1.0 mg./min.	9 min.	6.2	2.8	214%

[1] One unit per minute for ten minutes.

[2] $\frac{\Delta F \text{ after AMP}}{\Delta F \text{ after ACTH}} \times 100.$

ard "peaks" obtained from papers run with known amounts of the reference standards. This technique yields recoveries averaging 95% (range 88–102).

RESULTS

The effects of infusing 3′,5′ AMP into the arterial blood perfusing the intact isolated adrenal glands of 3 hypophysectomized and 3 nonhypophysectomized dogs are shown in Table 1. The blood flow into the adrenals was kept constant at 10 ml./minute, so that the effective concentration of 3′,5′ AMP (dissolved in 1 ml. 0.9% NaCl) added to this arterial blood was either 2.9×10^{-4} M (1.0 mg./min.) or 2.9×10^{-5} M (0.1 mg./min.). The secretion of hydrocortisone (cpd F) into the adrenal venous effluent was very low during the control collection periods in the hypophysectomized preparations, indicating virtual absence of endogenous ACTH, and variably high in the non-hypophysectomized preparations. Because of the variability in the responsiveness of the adrenals to the supramaximal dose of ACTH of one unit per minute from one dog to the next, we have tabulated the results in terms of the increment above control (ΔF) in hydrocortisone secretion following 3′,5′ AMP and ACTH. It can be seen from Table I that in 5 of 6 experiments the stimulation of cpd F secretion following 3′,5′ AMP was equal to or greater than stimulation following ACTH. Since hydrocortisone concentrations in the adrenal venous effluent of these six experiments were determined by a chemical procedure (10) which does not distinguish between cpd F and 11-desoxyhydrocortisone (cpd S), additional samples of the plasma were subjected to paper chromatography and it was found that both ACTH and 3′,5′ AMP primarily enhanced cpd F secretion. Small amounts of cpd B, which had not been detected in the control samples, were also found.

TABLE 2. COMPARISON EFFECT OF 3′,5′ AMP ON PRODUCTION OF CORTICOIDS BY SECTIONED RAT AND GUINEA PIG ADRENALS IN VITRO

Preincubation: 1.0 hour in Krebs-Ringer-bicarbonate-0.01 M glucose medium (1.2 ml. per 100 mg. wet tissue). Final incubation: 1.0 hour in the same medium. The control group of glands received no addition to the medium. One third of the glands received 7.5 μmoles of 3′,5′ AMP per ml. of medium in the rat experiment and 22.2 μmoles/ml. in the guinea pig experiment. The other third of the glands received 0.1 unit ACTH per 100 mg. of tissue in both experiments.

Addition	Corticoid output*
Experiment 1. (28 rats)	B μg/100 mg. wet tissue
None	1.8
7.5 μmoles 3′,5′ AMP	5.8
83 mu ACTH	6.2
Experiment 2. (15 guinea pigs)	F μg/100 mg. wet tissue
None	0.37
22.2 μmoles 3′,5′ AMP	0.73
83 mu ACTH	0.78

* As blue tetrazolium reacting material.

In every experiment of this group upon addition of the nucleotide to the blood perfusing the glands there was an immediate increase in the production of hydrocortisone which persisted as long as the 3′,5′ AMP was given (nine to forty-two minutes).

Table 2 illustrates the data from experiments in which 3,′5′ AMP and ACTH were added to rat and guinea pig adrenal slices incubated *in vitro*. The conditions of incubation and media used were as described by Haynes *et al.* in their experiments with rat adrenals (1, 4). In confirmation of the work of these investigators we noted increased synthesis of cpd B by the rat adrenal slices under the influence of 3′,5′ AMP at a concentration of 7.5×10^{-3} M. There was no cpd F detected in any of these experiments on the rat adrenal slices. In contrast, the guinea pig slices produced cpd F upon incubation with 3′,5′ AMP but only when a concentration of the nucleotide of 2.2×10^{-2} M was used. Small amounts of cpd B were also detected following both ACTH and 3′,5′ AMP.

Finally, in Table 3 are shown the results of four experiments in which norepinephrine and epinephrine secretion from the perfused isolated adrenal glands was measured. It can be seen that neither ACTH nor 3′,5′ AMP influenced catechol amine secretion from the adrenal medulla.

TABLE 3. LACK OF EFFECT OF 3′,5′ AMP AND ACTH ON NOREPINEPHRINE AND EPINEPHRINE SECRETION FROM ISOLATED PERFUSED DOG ADRENAL GLANDS

The amounts of ACTH and 3′,5′ AMP administered were the same as in experiments 1–4 of Table 1.

Procedure	No. periods	Norepinephrine μg/min.	Epinephrine μg/min.
Control	13	0.12	0.43
3′,5′ AMP	17	0.09	0.26
ACTH	11	0.16	0.45

DISCUSSION

Haynes and co-workers (3, 4) working with *in vitro* systems were the first to show that ACTH stimulation of corticosteroid production was mediated via 3′,5′ AMP activation of adrenal phosphorylase. In such systems, however, physiologic enzyme activities may be altered, reversibility of reaction may occur due to accumulation of reaction products, and temporal relationships of enzyme reactions may not be clearly definable if an equilibrium state is established. These considerations become important in view of the recent observation that in *in vivo* systems phosphorylase probably acts unidirectionally to produce continuous glycolysis (5).

It was therefore of interest to us to examine the effect of perfusion of 3′,5′ AMP in an *in vivo* system since this allowed for the study of the continuous effects of the nucleotide on the adrenal glands. If steroidogenesis were to proceed unabated during 3′,5′ AMP infusion, this would indicate that phosphorylase activation is an important factor in the response to ACTH. Our finding that perfusion of 3′,5′ AMP into the intact dog adrenal glands causes continuous production of corticosteroids in the absence of ACTH substantiates this concept.

Another feature of the present investigation is the demonstration of a species difference in the sensitivity of the response of the adrenal cortex to stimulation by 3′,5′ AMP. We have confirmed the work of Haynes *et al.* (4) that the rat adrenal slice incubated with 7.5×10^{-3} M concentration of 3′,5′ AMP responds with an increase in cpd B production of the same order of magnitude as that seen following incubation with ACTH. However, the Hartley strain guinea pig, a species in which cpd F is the major steroid produced after ACTH stimulation (6) only responds to the nucleotide at a concentration of 2.2×10^{-2} M. Whether this represents an altered cell permeability to 3′5′ AMP[3] cannot be answered from the present experiments. It should be noted, however, that the efficiency of steroidogenesis by the guinea pig adrenal either during control incubation or after ACTH is less than that by rat adrenal slices incubated under the same conditions (Table 2).

It is of interest to note that the perfused dog adrenal preparation appears to be the most sensitive to 3′,5′ AMP. We obtained good stimulation of hydrocortisone secretion at concentrations of 2.9×10^{-5} M of 3′,5′ AMP. A similar increased sensitivity to ACTH was noted by Macchi and Hecter (5) comparing perfused intact cow adrenal glands with incubated cow adrenal slices.

Since it has been shown that glucagon will stimulate the production of 3′,5′ AMP by liver slices (15) and that glucagon will also stimulate the

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[3] In one additional study, designed similarly to Experiment 2 of Table 2 except that guinea pig adrenal gland homogenates instead of slices were used, no stimulation of cpd F production was observed by either ACTH or 3′,5′ AMP.

secretion of epinephrine and norepinephrine by the isolated intact perfused dog adrenal gland (16) it was anticipated that 3′,5′ AMP would likewise stimulate catechol amine secretion. However, as seen in Table 3, dosages of 3′,5′ AMP and ACTH which produced good secretion of hydrocortisone failed to stimulate the adrenal medulla. The mechanism of stimulation of catechol amine synthesis would thus appear to be unrelated to nucleotide activity and still remains to be elucidated.

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THYROID REGULATORY FUNCTION OF INTRA-OCULAR PITUITARY TRANSPLANTS[1]

AZMI KHAZIN[2] AND SEYMOUR REICHLIN

Departments of Medicine, Preventive Medicine and Psychiatry, Washington University, St. Louis, Missouri

ABSTRACT

Thyroid regulatory responses to thyroxine administration and to hemi-thyroidectomy have been studied in hypophysectomized rats bearing intra-ocular pituitary transplants. The intra-ocular graft, though separated from direct contact with the hypothalamus stimulated thyroid I^{131} uptake but did not influence other measures of thyroid function (I^{131} release rate, thyroid weight, PBI). The grafts released ACTH but had no effect on growth or on testicular weight. In the presence of the graft but not in ungrafted hypophysectomized animals hemi-thyroidectomy was followed by increased TSH release as evidenced by a more rapid I^{131} release rate, stimulation of I^{131} uptake and thyroid enlargement. Thyroxine injection led to thyroid inhibition both in pituitary graft-bearing hypophysectomized and in normal animals, but not in ungrafted hypophysectomized rats. These findings were taken to indicate that pituitary tissue is capable of appropriate and autonomous TSH secretory responses to alterations in blood thyroid hormone level. It was also shown that thyroxine treatment depresses thyroid function to a greater degree than does hypophysectomy. The low I^{131} release rate of hypophysectomized rats is not further reduced by thyroxine. In contrast, pituitary graft bearing hypophysectomized rats (whose resting thyroid release rate is the same as those animals without the pituitary graft) have a marked thyroid inhibitory response to thyroxine. Maximum decrease in thyroid function after thyroxine administration thus appears to require the presence of pituitary tissue.

THE delicately balanced relationship that exists between the secretion of pituitary thyrotrophin (TSH) and the secretion of the thyroid hormone has been amply documented (1, 2, 3). Implicit in the assumptions of earlier workers in this field was that the circulating thyroid hormone acted directly on the anterior pituitary gland to regulate its secretion, but in more recent years attention has been increasingly drawn to the possibility that the nervous system, particularly the hypothalamic area is also involved in the response to altered blood thyroid hormone levels (1–5).

In a previous experiment it was found that massive hypothalamic destruction in the rat, though it reduces the level of thyroid function, does not prevent appropriate thyroid responses to thyroxine injection or to

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partial thyroidectomy (3). On the basis of this and other findings it was postulated that the pituitary is autonomously responsive to changes in thyroxine concentration and that its level of responsiveness is determined by a hypothalamic mechanism acting through the pituitary stalk.

In the present experiments thyroid regulatory functions of pituitary tissue removed from direct hypothalamic influences have been further studied by using hypophysectomized rats bearing intra-ocular pituitary transplants. As shown by Goldberg and Knobil (6) and by Martini *et al.* (7), these grafts become well vascularized, grow and maintain thyroid function at levels above that found in hypophysectomized animals. They are thus suitable preparations for the determination of the autonomous responsiveness of isolated pituitary tissue to alterations in blood thyroxine levels. A preliminary abstract of this work has been published (8).

METHODS

Hypophysectomized[3] male rats of the Sprague-Dawley strain, 175 to 224 gm. in weight, served as the recipients of the pituitary grafts. Between 3 and 14 days after hypophysectomy a pituitary from a fetal Sprague-Dawley rat[4] was inserted into the anterior chamber of each eye through a small medial corneal nick. The eyelids were kept closed by suture for three days to protect the wound. The donors were of either sex and were used at any time from 17 days after gestation to birth. In a few instances pituitaries from animals up to 36 hours after birth were used with satisfactory "takes." The recipient animals lived in a temperature controlled room at $26 \pm 1°$ C, were fed Purina laboratory chow supplemented with bread and oranges and were given tap water *ad libitum*. Control hypophysectomized and control normal rats were prepared with intra-ocular muscle transplants. Seventeen days after transplantation the animals were injected with 30 μc of I^{131} intraperitoneally; 24 hours later radioiodine uptake was measured by an *in vivo* counting method (9). Uptake values were calculated as percentage uptake per mg. thyroid tissue, the weight of the thyroid being determined either at the end of the experiment or estimated from the weight of the half removed at the time of hemi-thyroidectomy (*vide infra*). The rate of I^{131} release was determined by serial neck counts over the next four days and the rate of release in percent per day calculated from graphical plots on semi-logarithmic paper (Fig. 1). At all time intervals correction for extra-thyroidal neck radioactivity was made by counting the neck region of a group of 5 totally thyroidectomized animals. At the time of completion of the first release curve the right lobe of the thyroid gland was removed under ether anesthesia as previously described (10), and the weight of the removed portion measured on a torsion balance after clean dissection. Twelve days after the operation a second injection of an identical dose of I^{131} was given and the uptake and release rate again measured. Additional groups of engrafted rats were subjected to sham hemi-thyroidectomy. The response to thyroxine was tested by administering 50 μg. of l-thyroxine intraperitoneally on the fifth day of the second I^{131} release experiment. Following thyroxine injection, serial measurements of neck radioactivity were made for three additional days. Six days after the end of the experiment the animals were exsanguinated from the abdominal aorta under ether anesthesia, the thyroid remnant was removed and the degree of regeneration assessed by a comparison of the weight of the first removed and the second removed halves of the

[3] Hormone Assay Laboratories, Chicago.

[4] Holtzman Company, Madison, Wisconsin.

thyroid gland. Adrenal gland and gonad weight were also determined at death. Growth was estimated from measurements of body weight at the time of the transplantation and at death and by measurement of body length (nose-tail) at death. The eyes of the animals and the sellar region of the skull were fixed in 10% formaldehyde. The eyes were imbedded in celloidin, sectioned at 20μ and stained with hematoxylin and eosin. After decalcification in formic acid-formate solution the base of the skull was similarly imbedded and sectioned serially at 20μ for examination of the completeness of the hypophysectomy. Animals in which hypophysectomy was incomplete would have been discarded from the series but none was found. Plasma obtained at death from the sham operated normal and engrafted animals was analyzed for protein bound iodine[5] (PBI), as was the plasma from those thyroidectomized rats which had been used in the experiment to correct for extra-thyroidal neck radioactivity.

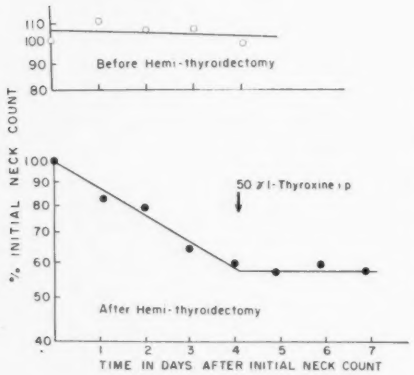

FIG. 1. This figure illustrates the effects of hemi-thyroidectomy and of thyroxine injection on I^{131} release rate in a hypophysectomized rat bearing an intra-ocular pituitary transplant. The initial rate of approximately 4% per day may be contrasted with the normal rate of approximately 25% per day. Hemi-thyroidectomy was followed by acceleration in release rate, thyroxine by prompt and dramatic inhibition of I^{131} release.

RESULTS

The Thyroid Gland in Hypophysectomized Rats Bearing Intra-ocular Pituitary Transplants

Following hypophysectomy there was a marked reduction in thyroid size, I^{131} uptake, I^{131} release rate and plasma PBI (Table 1). The pituitary grafts stimulated thyroid activity only slightly. As compared with non-grafted hypophysectomized rats I^{131} uptake was significantly elevated while the other measures of thyroid stimulation (release rate, thyroid size and PBI) though slightly higher were not significantly so by statistical test. The PBI of surgically thyroidectomized rats was not different from that of the hypophysectomized animals.

Response to Hemi-thyroidectomy

Following hemi-thyroidectomy both normal and graft-bearing hypophysectomized rats manifested thyroid activation as evidenced by highly significant increases in I^{131} release rate, thyroid remnant size, and I^{131} uptake per mg. of thyroid tissue (Table 2, Fig. 1). The changes in I^{131} uptake

[5] Bio-Science Laboratories, Los Angeles, California.

TABLE 1. THYROID FUNCTION IN HYPOPHYSECTOMIZED RATS BEARING INTRA-OCULAR PITUITARY TRANSPLANTS

Group	No. of animals	Time after transplant (days)	I^{131} Uptake (% of dose per mg. thyroid) ±S.E.	I^{131} Release rate %/day ±S.E.	Thyroid size mg. ±S.E.	PBI μg% ±S.E
I. Hypox+pituitary transplant	10	17	0.21 ±.03	3.9 ± 0.8	8.7[1,2] ±0.4	
II. Hypox+muscle transplant	3	17	0.11	4.2	8.6[1,2]	
III. Normal+muscle transplant	5	17	0.39 ± .04	24.0 ± 1.9	16.6[1,2] ±0.5	
IV. Hypox+pituitary transplant	9	37	0.21 ± .04	4.6 ± 0.3	8.2[3] ± 0.4	1.8 ±0.1
V. Hypox+muscle transplant	4[4]	37	0.12	4.0	8.0[3]	1.6
VI. Normal+muscle transplant	5	37	0.60 ± .06	22.8* 2.1	14.5[3] ± 0.6	3.8 ±0.1
VII. Total thyroidectomy	4					1.6 ±0.1
*Statistical Comparison (P values)**						
Pituitary transplants vs. muscle transplant (Groups I+IV vs. II+V).			<.05	<.7	<.8	
Pituitary transplants vs. normal (Groups I+IV vs. III+VI)			<.001	<.001	<.001	
Muscle transplants vs. normal (Groups II+V vs. III+VI)			<.001	<.001	<.001	
Pituitary transplants vs. normal (Group IV vs. VI)						<.001

[1] Thyroid weight 25 days after transplant.
[2] Estimated from weight of left lobe.
[3] Thyroid weight 48 days after transplant.
[4] 3 animals for PBI determination.
* Calculated by method described by Snedecor (24).

and release occurred in each individual animal of these groups. In controls the three muscle-bearing hypophysectomized animals suffered a fall in uptake and scarcely detectable changes in I^{131} release rate and in remnant weight. That the observed accelerated thyroid activity was in fact a consequence of hemi-thyroidectomy is indicated by the changes in animals subjected to the sham procedure; in these rats I^{131} release rate showed no significant change over the period of observation.

Response to Thyroxine Injection

Every one of the normal and 16 out of the 19 hypophysectomized rats bearing intra-ocular pituitary transplants manifested marked or complete inhibition of I^{131} release following a single injection of 50 μg l-thyroxine

TABLE 2. THYROID RESPONSE TO HEMI-THYROIDECTOMY IN HYPOPHYSECTOMIZED RATS BEARING INTRA-OCULAR PITUITARY AND MUSCLE TRANSPLANTS

Group	No. of animals	Time after thyroidectomy (days)	Percentage change in I^{131} uptake (per mg. thyroid)		Change in I^{131} Release rate*		Percentage change in remnant weight	
			%	±S.E.	%	±S.E.	%	±S.E.
Hemi-thyroidectomized								
I. Hypox+pit. transplant	10	12	+54.5	15.6	+8.6	1.4	+22.6	2.1
II. Hypox+mus. transplant	3	12	− 5.5		+0.5		+ 1.0	
III. Normal+mus. transplant	5	12	+25.5	7.9	+6.6	1.0	+34.6	1.7
Sham Hemi-thyroidectomy								
IV. Hypox+pit. transplant	9	12			−0.8	0.5		
V. Hypox+mus. transplant	4	12			−0.6			
VI. Normal+mus. transplant	5	12			−0.4	0.2		
			*Statistical Analysis (P values)***					
Changes after Hemi-thyroidectomy								
I			<.01		<.001		<.001	
III			<.05		<.01		<.001	
I vs. III			<.3		<.4		<.01	
Changes after Sham Operation								
IV					<.2			
VI					<.2			
IV vs. VI					<.6			

* Value given is the arithmetic difference between the slopes of pre- and post-hemithyroidectomy curves. This mode of expression of change in slope was adopted in order to avoid the calculation of percentage increase which is misleading when initial release rate values are very low. A relatively small and insignificant difference in initial release rate, i.e., 0.2 vs. 0.4% per day, can give almost a twofold difference in calculated percentage increase.

** Calculated by method described by Snedecor (24).

i.p. (Fig. 1, Table 3). In contrast, release rates in hypophysectomized animals without pituitary grafts were unaffected by thyroxine injections, even though baseline rates in these animals were not significantly different from release rates in the sham thyroidectomized animals with pituitary grafts. It is apparent moreover, that the I^{131} release rate of thyroxine-treated normal rats is significantly slower than the release rate of hypophysectomized rats with or without thyroxine treatment ($P < .001$).

Growth, Adrenal and Gonad Size (Table 4)

From weight and length measurements there was no evidence that the pituitary grafts stimulated growth, although all hypophysectomized rats did display slight increases in body weight. The testes were slightly heavier in rats bearing intra-ocular grafts, but the difference was not significant statistically. The adrenals by contrast were significantly heavier in rats with transplants but they did not attain normal size.

Histological Findings

Examination of sections of the eye in rats with intra-ocular pituitary transplants indicated excellent growth of pituitary tissue, and as revealed

TABLE 3. EFFECT OF THYROXINE ON I^{131} RELEASE RATE IN NORMAL, HYPOPHYSECTOMIZED AND PITUITARY-ENGRAFTED HYPOPHYSECTOMIZED RATS

Group	No. of animals	I^{131} release rate				Significance of difference (P value)*
		Before thyroxine		After thyroxine		
		%/day	S.E.	%/day	S.E.	
Hemi-thyroidectomized						
I. Hypox+pituitary transplant	10	12.4	1.0	0	0	<.001
II. Hypox+muscle transplant	3	4.7		4.7		
III. Normal+muscle transplant	5	30.6	1.7	0.2	.1	<.001
Sham Hemi-thyroidectomized						
IV. Hypox+pituitary transplant	9	4.6	.3	1.5	.7	<.001
V. Hypox+muscle transplant	4	4.0		4.0		
VI. Normal+muscle transplant	5	22.8	2.1	0.3	.3	<.001
Hypophysectomized+muscle transplant taken together (Groups II & V)	7	4.3	.8	4.3	.8	1

* Calculated by method described by Snedecor (24).

by India ink injections, good blood supply. There was a notable decrease in the size of the posterior lobe and an apparent increase in the width of the intermediate lobe. Because of the nature of the imbedding material specific histochemical stains were not applied so that no comments can be made about the nature of the cells found in the ocular transplant. The blood supply to the transplant was seen to come chiefly from vessels arising in the ciliary body and iris.

DISCUSSION

In this experiment it has been shown that the pituitary, though transplanted to the anterior chamber of the eye, is capable of stimulation of

TABLE 4. GROWTH, GONAD AND ADRENAL WEIGHT IN RATS WITH INTRA-OCULAR PITUITARY TRANSPLANTS (7 WEEKS AFTER GRAFT)

Groups	No. of animals	Body weight			Body length at death		Testicular weight		Adrenal weight	
		Initial	Increase %	±S.E.	cm.	±S.E.	gm.	±S.E.	mg.	±S.E.
I. Hypox+pit. transplant	19	195	4.2	1.0	37.6	0.0	0.52	0.02	16.1	0.5
II. Hypox+muscle transplant	7	192	5.6	2.0	37.7	0.3	0.48	0.02	12.3	0.7
III. Normal control	10	322	19.4	2.0	43.3	0.2	3.34	0.13	46.0	1.5
Statistical Analysis (P values)*										
I			<.001							
II			<.001							
III			<.001							
I vs. II			<.5		<.9		<.4		<.001	
I vs. III			<.001		<.001		<.001		<.001	

* Calculated by method described by Snedecor (24).

the thyroid gland. Uptake is increased over the level observed in control hypophysectomized rats, a finding confirmatory of that of Goldberg and Knobil (6) who in similar preparations measured thyroid/serum ratio (T/S ratio). The observation of Martini *et al.* (7) that intra-ocular grafts stimulate 4-hour I^{131} uptake in hypophysectomized rats is similarly confirmed. Unlike the findings of the above cited authors, significant increase in thyroid weight was not observed. I^{131} release rate was not elevated by the transplant, a result in contrast to that of von Euler and Holmgren (11) who have demonstrated a substantial rate of I^{131} release in the hypophysectomized rabbit after pituitary grafting. The apparent contradiction in findings is probably of little importance because of the varying sensitivity of the various parameters of TSH stimulation (12, 13) under differing conditions in the animal with very low levels of thyrotrophin activity. Taken as a whole, these findings may be interpreted to indicate that the intra-ocular pituitary transplant exerts a modicum of TSH function.

Numerous factors contribute to the relatively low thyrotrophic activity of intra-ocular grafts. Blood supply and hence blood flow of the graft in the eye are manifestly poorer than that of the normally placed gland and the function of two fetal pituitaries in these studies is being compared with that of a normal adult pituitary; qualitative and quantitative differences in hormone secretion may be present. Furthermore, as indicated by the elegant studies of Nikitovitch-Winer and Everett (14) the site of transplant is of importance in determining function. Grafts under the median eminence of the rat were found to stimulate the thyroid gland to a greater degree than did grafts in the kidney or under the temporal lobe.

From data presented it may also be concluded that the isolated pituitary is capable of autonomous responses to change in thyroxine environment, although the resting level of activity is reduced far below the normal. Thyroxine injection significantly lowered I^{131} release rate in the rat transplant preparation, a finding confirmatory of that of von Euler and Holmgren who have similarly studied the rabbit (11). This result was also to be expected from the pituitary microinjection experiments of these workers (15) and of Yamada and Greer (16).

As indicated by the significant changes that occur in I^{131} uptake and release rate and by the significant (though small) changes in thyroid remnant weight after hemi-thyroidectomy the isolated pituitary appears to be capable of discharge of TSH in response to lowered blood thyroid hormone levels. Added support for this view comes from Scow and Greer (17) who demonstrated an increase in iodide-trapping capacity of the thyroid gland following the feeding of propylthiouracil to hypophysectomized mice bearing intra-ocular transplants.

The pituitary transplanted to a site remote from the hypothalamus, like that of the rat with extensive hypothalamic damage (3), thus demonstrates qualitatively normal responses to change in thyroxine environment

despite its operation at a markedly reduced baseline level of function. The same probably holds true for the pituitary in the stalk sectioned rabbit (18), at least as far as the effects of thyroxine injection are concerned. From the observation that the rat with a pituitary transplant and the rat with extensive hypothalamic damage both display low levels of TSH activity yet appear capable of increasing their secretion when thyroxine levels are further reduced (as by hemi-thyroidectomy or by propylthiouracil feeding) it seems reasonable to believe that the threshold of response to a given thyroxine concentration has been altered in these animals. The mechanism of alteration in threshold may be rationalized by the previously suggested hypothesis (3) that the pituitary itself acts as the thyrostat, the setting of which is governed by a hypothalamic mechanism. This view complements rather than conflicts with the excellent work of Yamada (2) and of Yamada and Greer (16) who present evidence for a local effect of thyroxine on the hypothalamus as well as on the pituitary.

Consideration must be given to the possibility that the results noted in this experiment were due to the release of a circulating hormone from the hypothalamus which led to activation of the transplant by way of the peripheral circulation. A definitive test of this hypothesis would be the repetition of a similar experiment in hypophysectomized animals with hypothalamic damage in the "thyrotrophic" area. Such studies are now underway in this laboratory.

Preservation of the qualitative thyroid feedback response in the grafted rat is analogous to the function of the pituitary-adrenal system in rats with kidney grafts. As recently shown by Hertz (19), characteristic adrenal hypertrophy follows amphenone administration to these preparations. Interestingly, Hertz could not demonstrate the normal goitrogenic effect of this drug, a finding undoubtedly analogous to the "goiter block" produced by hypothalamic lesions (4). That the pituitary graft releases detectable amounts of ACTH is clear from the present study which is also confirmatory of other investigations (6, 7, 19). Growth promoting and testicular stimulating effects of the grafts are less well established. In this experiment and that of Martini *et al.* (7), neither body growth nor testicular growth were observed in graft-bearing animals, a finding at variance with that of Goldberg and Knobil (6). Stimulation of growth by pituitary transplants to the kidney has been observed by Hertz (20). The significance of these differences is not clear but they may in part be related to the volume of viable pituitary tissue, the age of the donor, the age of the host, or to host-graft immunological factors.

An incidental finding of great interest was that thyroxine reduced I^{131} release rates in the normal animals to levels considerably lower than the rate produced by hypophysectomy. When administered to hypophysectomized animals without pituitary grafts thyroxine did not affect the release rate; when given to hypophysectomized animals with pituitary grafts,

thyroxine induced a further lowering of the already reduced rate. This difference of effect of thyroxine on pituitary-grafted as compared with muscle-grafted hypophysectomized rats could not be explained by differences in pre-treatment release rates because these were almost identical in one of the series studied. Therefore pituitary tissue must be present in order to obtain the maximum inhibitory effects of thyroxine on the thyroid gland. The recent studies of Halmi *et al.* (21) are highly relevant to these observations. Triiodothyronine (T3) administered to normal rats on a thiouracil containing diet was found to reduce the thyroid/serum iodide ratio (T/S ratio) to mean values between 1.0 and 2.7. Hypophysectomized rats on a similar diet had mean T/S ratios between 5.9 and 7.3. The administration of T3 to the hypophysectomized rats did not lead to a further depression in T/S ratio. Halmi *et al.* (21) conclude that T3, in addition to its effect in inhibiting TSH release must exert an effect on the thyroid which is "either mediated through the hypophysis or depends on the permissive action of one or more pituitary or target gland principles." These authors question the physiological significance of their findings because the rats were receiving thiouracil, large doses of T3 and cortisone. In the present studies results analogous to those of Halmi were observed without these complicating features and with a single injection of 50 μg of l-thyroxine. The physiological significance of these results nevertheless remains uncertain.

The early observations and discussion by Reforzo-Membrives (22) come to mind in considering an explanation of this problem. This author asserted that the pituitary of thyroid-fed rats contains a substance which, upon injection into guinea pigs, reduces thyroid weight and follicular cell height and lowers the BMR. The existence of a thyroid inhibitory hormone(s) was thus postulated. This work has not been confirmed, and a detailed criticism of the techniques and conclusions has been published (23). Reinvestigation of the question of thyroid inhibitory substances of pituitary origin seems clearly indicated in view of the present studies and those of Halmi and his group (21). There is an equally good possibility that I^{131} metabolism in the thyroid of hypophysectomized rats is qualitatively different in the presence of the small amount of TSH released from the graft, even though overall I^{131} release rate is the same as in the animal without the graft. A detailed comparison of iodide handling by the thyroids of normal, hypophysectomized and thyroxine-treated normal rats is under current investigation in this laboratory.

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CERTAIN FUNCTIONS OF ISOLATED THYROID CELLS

IRA PASTAN

Clinical Endocrinology Branch, National Institute of Arithritis and Metabolic Diseases, National Institutes of Health, Bethesda, Maryland

ABSTRACT

A technique for dispersing thyroid tissue into single cells is described. When incubated with I^{131} these cells form almost entirely monoiodotyrosine in peptide linkage. Iodination is stimulated by TSH and inhibited by propylthiouracil, thiocyanate, catalase and glutathione. Perchlorate is without effect. Thyroglobulin, serum albumin and tyrosine are iodinated when added to the incubation medium, although the iodinated material formed by unfortified cells does not appear to be thyroglobulin. These cells actively metabolize glucose to CO_2 and oxidize glucose by way of the hexose monophosphate pathway.

THE thyroid gland possesses follicles where hormone is stored in large amounts. Which steps in the biosynthesis of thyroid hormone occur in the cell and which occur in the colloid are not known. Autoradiographic evidence suggests that the formation of organic iodine compounds occurs within the thyroid follicle just at its boundary with the thyroid cell, but not in the cell (1). Iodide also appears to be concentrated in the follicle and not in the cell (2) (3). This study on the iodine metabolism of dissociated thyroid cells incubated with I^{131} for relatively short periods of time has been initiated to investigate the functional ability of the thyroid cell separated from its neighbors and deprived of its colloid. Pulvertaft *et al.* have reported that isolated thyroid cells grown in tissue culture in the presence of I^{131} form only very small amounts of iodinated tyrosines, thyroxine or triiodothyronine (4).

MATERIALS AND METHODS

Calf thyroids were obtained from the local abattoir, wrapped in parafilm and kept on cracked ice until used. Numerous methods of preparing dispersed cells were investigated and a modification of the method of Younger was found most satisfactory (5). Twenty grams of trimmed glands were sliced with the aid of a Stadie-Riggs slicer. The slices were rinsed in Krebs-Ringer bicarbonate buffer (pH 7.4) and then transferred to a small Waring blender containing 60–70 cc. of Krebs-Ringer bicarbonate buffer made up without calcium or magnesium. In this were dissolved crystalline trypsin and sodium citrate to concentrations of 0.4% and 0.1% respectively. The tissue was then incubated at 37° C for 15 minutes and swirled at low speed at this temperature for a further 15 minutes. The speed was adjusted to produce vigorous agitation without foaming or cutting of the slices. The cloudy supernatant was decanted through four layers of fine gauze and

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chilled, while the remaining slices were reincubated in the blender with fresh media five additional times. There was a tendency for the slices to adhere and they were separated before some incubations by briefly mincing with a sharp scissors. The cells were harvested from the supernatant by spinning for 3½ minutes at 1400 G. The button was suspended in complete Krebs-Ringer bicarbonate buffer containing 200 mg.% glucose and 50 mg. of soybean trypsin inhibitor to inactivate any remaining trypsin. The cells were again sedimented and washed twice more in buffer without trypsin inhibitor to get rid of all possible contaminating subcellular particles. The final button was a gelatinous mass weighing 0.6 to 1.0 gm. It was composed of thyroid cells, a variable number of red cells and some nuclei. On numerous occasions a portion of this final preparation of cells was dispersed in saline and examined unstained with the light microscope. Although 1% of the cells were in clumps of two or rarely three, the rest were single cells. An intact follicle was never seen.

The button of cells obtained from the final centrifugation was divided into six or eight equal portions, weighed and transferred to 25 ml. Erlenmeyer flasks containing 3 ml. of Krebs-Ringer bicarbonate buffer (pH 7.4), 200 mg.% glucose, 25 microcuries of I^{131} and 0.1 μg of sodium iodide to insure constant iodide levels. Test substances were dissolved in buffer. The flasks were shaken for 2 hr. at 37° C on a Dubnoff metabolic shaker with air as gas phase. The pH at the end of incubation was 7.8. A gas phase of 95% oxygen and 5% CO_2 was also satisfactory. The button of cells initially placed in each flask dispersed within 10 minutes. However, at the end of the incubation the cells were once more clumped together in a thin string 1–2 cm. long. Fifty microliters of 10^{-2} Molar propylthiouracil (PTU) were added at the end of the incubation and the contents of the flasks frozen, thawed and homogenized to disperse the iodinated products uniformly. One half ml. aliquots were digested with pancreatic extract (Viokase) at 37° C for 20 hours under toluene (pH 7.8). Aliquots were chromatographed in n-butanol: acetic acid: water (75-10-15) and n-butanol: dioxane (8-2) saturated with 2N NH_4OH. Two dimensional chromatography was performed when indicated. Carrier compounds were always added to chromatograms to facilitate the identification of radioactive spots. Radioactivity was measured with an end window Geiger-Muller counter employing a continuous recording, counting rate meter. In addition autoradiographs were performed on selected chromatograms. Results are the average of duplicate experiments done on three or more separate occasions which were in reasonably close agreement.

Ultracentrifugation was performed in 0.04 M NaCl in a Spinco Model L ultracentrifuge using a swinging bucket rotor at 85,000 G (average) for 45 minutes. Samples were first dialyzed against .001 Molar phosphate buffer until all iodide was removed. Paper electrophoresis was performed in barbital buffer, pH 8.6, ionic strength 0.1 at 3.3 volts/cm. for 20 hours. Strips were dried at room temperature, scanned with the strip counter and stained with bromphenol blue. Thyroglobulin was prepared by a modification of the method of Edelhoch (6). In experiments in which glucose oxidation was studied, weighed aliquots of cells were incubated for 45 minutes in 2.0 ml. of Krebs-Ringer bicarbonate buffer containing 5 mg. of glucose and 0.5 μc of either glucose-1-C^{14} or glucose-6-C^{14}. The reaction was stopped by the addition of 0.2 ml. of 10N H_2SO_4 and the $C^{14}O_2$ collected in 1 ml. hyamine base injected through a rubber stopper into a center well. The hyamine containing $C^{14}O_2$ was transferred to a counting vial, mixed with diphenyloxazole and toluene, and counted in a liquid scintillation counter.

Catalase, trypsin (1×crystalline), and soybean trypsin inhibitor, were obtained from Worthington Biochemical Corporation. The thyroid stimulating hormone (TSH) used was Thytropar (Armour). C^{14}-glucose was obtained from the National Bureau of Standards.

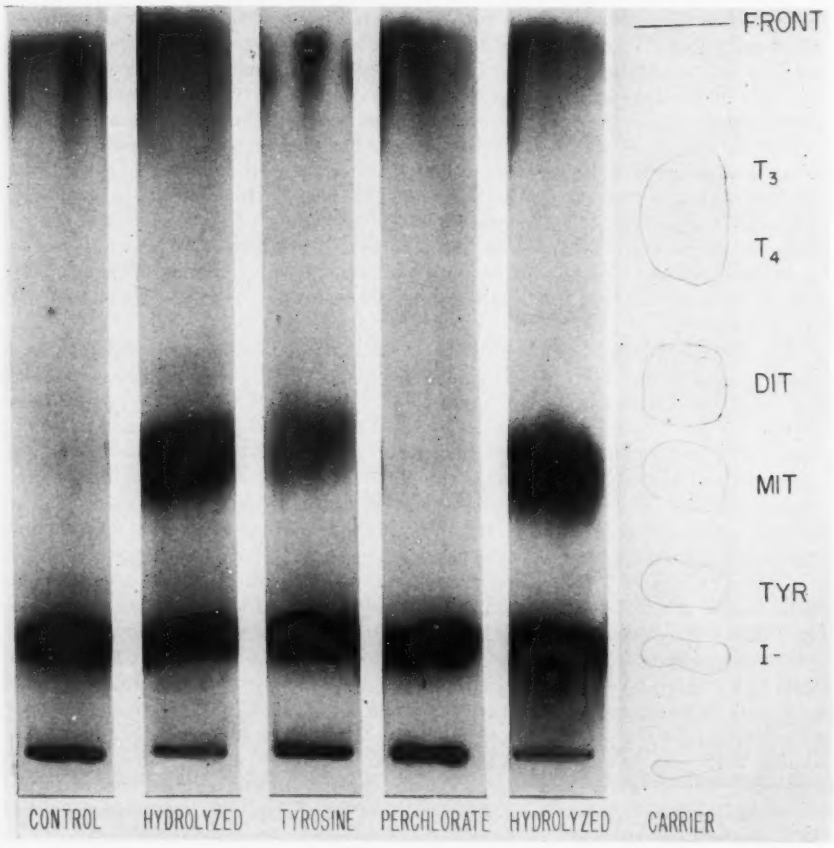

FIG. 1. Autoradiographs of chromatograms of iodinated compounds formed by isolated thyroid cells. Solvent is n-butanol: acetic acid: H_2O (75-10-15).

RESULTS

Thyroid cells can incorporate I^{131} into iodinated proteins which remain at the origin on chromatograms (Fig. 1). The formation of iodinated proteins increases with time and in one experiment had not reached a plateau at two hours (Fig. 2). In another experiment the plateau level was not approached for four hours. The amount of iodinated material formed was consistent within any one preparation of cells but varied from day to day, ranging from 5–60% of the radioactivity. In one experiment in which carrier iodide was omitted, the same percentage of iodinated material was formed as when the carrier was present. On the other hand, increasing the iodide up to 10 or 100 μg per flask decreased the percentage of iodinated proteins formed (Table 1). Usually not more than 10% of the radioactivity appeared at the solvent front. Application of a small clump of cells directly

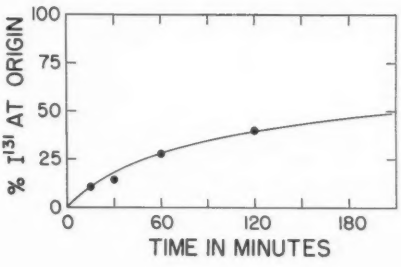

FIG. 2. I^{131} uptake of isolated thyroid cells.

to a chromatographic strip revealed more protein bound iodine in the cells than in an equal volume of medium.

The nature of the newly formed iodoproteins was investigated. Digestion with pancreatic extract released mainly monoiodotyrosine (MIT). On a few occasions up to 10% of the protein bound radioactivity was in the form of diiodotyrosine (DIT), but usually this was less than 1%. No thyroxine was identified although occasional unidentified radioactive spots were noted. Prolongation of incubation time to 8 hours and enrichment of the media with thyroglobulin, albumin, TSH, thyroid homogenate or human serum albumin did not increase the yield of DIT or result in the formation of thyroxine.

Thyrotropin stimulated I^{131} incorporation (Table 2). Addition of PTU, thiocyanate, catalase and reduced glutathione diminished or abolished I^{131} incorporation. Perchlorate, however, was without effect (Table 3). Tyrosine (10 μmoles) was iodinated to free MIT (Fig. 2). MIT (10 μmoles) and thyronine (10 μmoles) were inhibitory. Carrier-free MI^{131} T was not incorporated into iodinated protein, and there was little deiodination. This is somewhat surprising since thyroid slices actively deiodinate MIT. However thyroid homogenates require TPNH to deiodinate and these thyroid cells may be depleted of this cofactor. Neither 3,5 diiodothyronine (10 μmoles) nor histidine (10 μmoles) were iodinated.

Ultracentrifugation of the iodinated proteins revealed that 19–52% of them were soluble, the rest particulate. The percentage of the iodinated

TABLE 1. THE EFFECT OF SOME SUBSTANCES ON IODINATION BY THYROID CELLS

Experiment	Drug	Am't per flask	% Radioactivity at		
			Origin	Iodide	Front
1	Control	—	38	58	4
	MIT	10 μmoles	3	97	0
	Thyronine	10 μmoles	4	96	0
2	Control	—	22	78	0
	NaI	none	20	77	3
	NaI	10 μgms.	2	98.0	0
	NaI	100 μgms.	0.5	99.5	0

TABLE 2. STIMULATORY EFFECT OF TSH ON IODINATION BY THYROID CELLS

Experiment	Drug	Drug amount	% Radioactivity at			
			Origin	Iodide	Between iodide & front	Front
1	Control	—	21.5	68.5	—	10
	TSH	1 unit	39	55	—	6
2	Control	—	2	78	(8)	12
	TSH	1 unit	16	42	(8)	34
3	Control	—	36.5	59.6	(1.5)	2.4
	TSH	1 unit	58.5	33.2	(2.3)	6
4	Control	—	29	60	(4)	7
	TSH	.05 units	44	37	(6)	13

proteins that was soluble was increased by the addition of thyroglobulin or albumin. Examination of the particulate iodoprotein in the ultracentrifuge revealed that it sedimented into three zones.[1] The particulate iodoprotein of normal thyroid and a functioning rat thyroid tumor exhibited the same sedimentation characteristics (7). No attempt was made to solubilize this material.

Electrophoresis of the soluble iodoproteins formed revealed more than one peak migrating from the origin toward the anode. However, no peak migrated to the region of calf thyroglobulin. The addition of calf thyroglobulin or human serum albumin resulted in their iodination (Fig. 3). Catalase inhibited the iodination of serum albumin.

In three experiments 10^{-3} Molar PTU or methyl mercaptoimidazole was added to the media containing 25 μc of I^{131} before the cells were introduced. In two experiments 0.1 μg of I^{127} was present and absent in the third. After a two hour incubation there was no concentration of iodide by the cells. Radioautographs indicate that iodide is mainly concentrated in the colloid (2) (3). The thyroid cell may actively secrete iodide into the follicular lumen without the intracellular concentration being higher than that of the medium.

TABLE 3. THE EFFECT OF VARIOUS SUBSTANCES ON IODINATION BY THYROID CELLS

Drug	Drug concentration	% of Radioactivity at		
		Origin	Iodide	Front
Control	—	25	72	3
KSCN	10^{-3} M	3	97	0
PTU	10^{-3} M	0	100	0
$NaClO_4$	10^{-3} M	27	68	5
Catalase	1000 units	4	96	0
Glutathione	10^{-3} Molar	5	83	12
Boiled	—	0	100	0

[1] Kindly performed by Dr. David W. E. Smith.

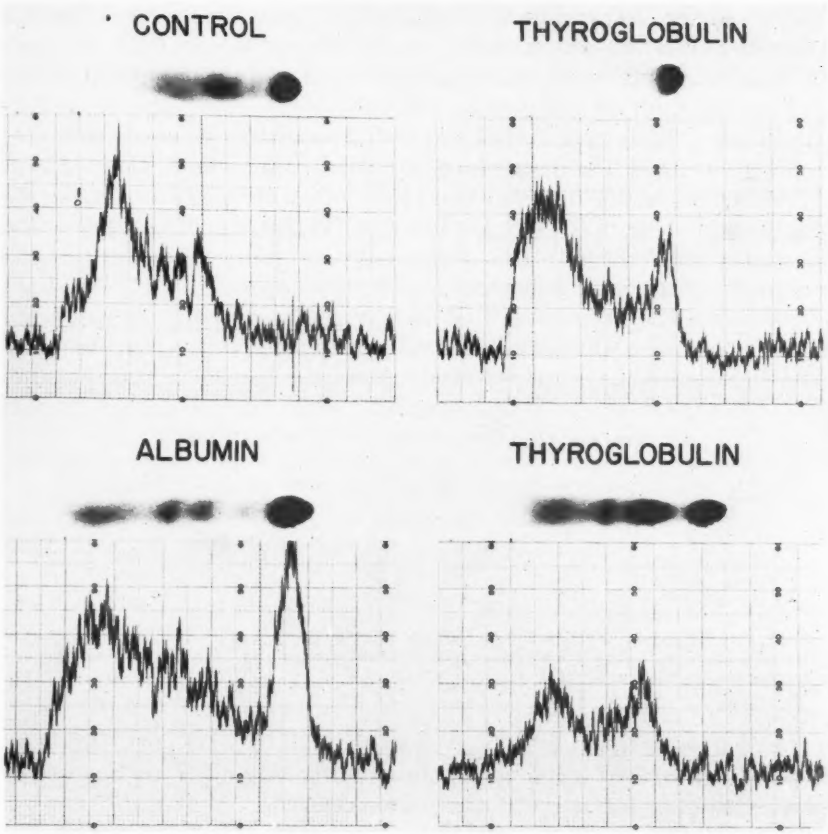

FIG. 3. Zone electrophoresis of iodoproteins formed by isolated thyroid cells. Carrier human serum was added before electrophoresis to all experiments except that shown at the upper right.

DISCUSSION

Dispersed thyroid cells free of colloid can be readily prepared by the method described. A survey of some of their metabolic functions revealed them to be qualitatively intact. Glucose-C^{14} can be metabolized to $C^{14}O_2$. In addition the presence of the hexose monophosphate pathway for glucose metabolism was evident on finding a ratio of 17/1 in comparing counts/gm./45 minute incubation of $C^{14}O_2$ derived from glucose-1-C^{14} with $C^{14}O_2$ derived from glucose-6-C^{14} (Table 4). Slices of calf thyroids oxidize glucose in a like manner although unfortified thyroid homogenates are unable to oxidize glucose to $C^{14}O_2$ (8) (9). Incubation of isolated cells with $Na_3P^{32}O_4$ revealed incorporation of P^{32} into phospholipids.[2]

[2] Kindly performed by Dr. P. Vilkki.

These dispersed thyroid cells incorporate I^{131} into soluble and particulate iodoproteins. Hydrolysis of these proteins releases mainly MIT although on some occasions small amounts of DIT are also seen. Thyroid homogenates also form an iodoprotein, but this appears to be almost entirely particulate (10). Tyrosine incubated with thyroid cells is converted to free monoiodotyrosine. The formation of free MIT has also been observed with thyroid homogenates fortified with CU^{++} or an H_2O_2 generating system plus tyrosine (11) (12) (13). Radioactive MIT is not incorporated into any iodoprotein suggesting that normally tryrosine is first synthesized into protein before it is iodinated.

The stimulation obtained with TSH seems to support the specificity of the iodinating ability of thyroid cells. Although *in vitro* stimulation of iodine metabolism has been previously reported, effects on glucose metabolism and phospholipids have been more striking (8) (14) (15). However, despite the increased iodide incorporated into iodoprotein, no increase in diiodotyrosine or formation of thyroxine occurred.

TABLE 4. GLUCOSE OXIDATION BY THYROID TISSUE

Tissue	$C^{14}O_2$ cpm/gm./45 minute incubation derived from	
	Glucose-1-C^{14}	Glucose-6-C^{14}
Slices	5,900	1,500
Cells	7,000	420
Homogenate	0	0

Each flask contained 500,000 counts/min. of glucose-1-C^{14} or glucose-6-C^{14}. Homogenate prepared by homogenizing 2.5 gm. of calf thyroid in 7 ml. of .15 M KCl. 1 ml. aliquots of homogenates added to each flask.

The ability of catalase to inhibit iodination by these relatively well organized cells strongly implicates H_2O_2 as the oxidizing agent for $I^- \rightarrow I_2$. Inhibition of iodination was also produced by glutathione and PTU. In addition to its effects as a peroxidase inhibitor and an antithyroid agent, PTU, like glutathione, is a reducing agent. On the other hand, perchlorate was not inhibitory. Since these cells lack the ability to concentrate iodide, perchlorate which seems only to inhibit iodide concentration, would not be expecte to interfere with organification. The fact that thiocyanate is an effective inhibitor suggests that at this concentration its action is different from that of perchlorate. This difference has also been noted in homogenate iodinating systems (16) (13), and may be due to its activity as a reducing agent.

Catalase (MW 248,000) is a large molecule and would not be expected to enter cells. Its ability to inhibit iodination suggests that iodination occurs at the cell membrane. This would agree with autoradiographic evidence indicating that iodination occurs in the follicle at its junction with the cell (1). The iodination of added thyroglobulin, human serum albumin

and tyrosine suggests that the iodinating mechanism is not specific for any one protein or even for a protein. In turn this raises the possibility that the reason thyroglobulin is the main iodinated protein in the thyroid is that it resides at the iodinating site, the colloidal side of the thyroid cell. If thyroglobulin is replaced by another protein such as serum albumin, then albumin is iodinated instead. This is supported by the observation that there are other iodinated proteins in the thyroid besides thyroglobulin.

The failure of these cells to synthesize very much diiodotyrosine and any thyroxine may mean that the enzymes and substrates necessary for the formation of these compounds lie in the follicular lumen. However, fortification with thyroid homogenate did not stimulate the formation of these compounds. Another reason for this failure might be that the mechanical agitation and trypsinization of the preparative procedure may have damaged cell membranes and leached the cells of important cofactors.

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ANTIPHLOGISTIC PROPERTY OF DESOXYCORTICOSTERONE AFTER PARTIAL HEPATECTOMY, ADRENALECTOMY, FORCE-FEEDING AND KCl INGESTION IN THE RAT

ANDRÉ ROBERT AND JAMES E. NEZAMIS

Department of Endocrinology, The Upjohn Co., Kalamazoo, Michigan

ABSTRACT

Desoxycorticosterone (DOC) has been shown to be antiphlogistic in the rat after both local and systemic administration. When given systemically, it is, in addition, thymolytic and catabolic. It was found that these new properties of DOC are greatly augmented after removal of 70% of the liver. The efficient inactivation of the steroid by the liver may explain why high doses must be given in intact animals.

The present investigations also indicate that the antiphlogistic and catabolic properties of DOC are specific and not due to stress reaction consequent to metabolic disturbances of the hormone. Actually, the effect observed was not modified by adrenalectomy, force-feeding or potassium chloride supplements.

The difference in activity between DOC and cortisol appears to be mainly in degree, the former being much weaker.

DESOXYCORTICOSTERONE has been shown to inhibit inflammation in the rat after both local (1) and systemic (2) administration. By the latter route, the steroid has to be given at rather high doses, from 5–10 mg. daily. Reichstein's compound "S" also has the same activity and is half to one-third as potent as desoxycorticosterone (2). Finally, progesterone is antiphlogistic locally, but much less active after systemic administration (2). Among the hypotheses proposed to explain the antiphlogistic property of these 11-desoxy steroids, two were retained for further investigation. First, it was thought that if high doses have to be administered systemically in order to produce the desired effect, it may be because these steroids are effectively handled by the liver and rapidly inactivated. The second hypothesis questioned the specificity of the antiphlogistic activity observed: since only high doses are antiphlogistic, the steroids may elicit some metabolic changes that are stressful to the animal. Stimulation of the hypophyseal-adrenal system would then inhibit inflammation. In the present communication, these two possibilities were studied, and desoxycorticosterone (DOC) was used.

1. *Systemic Effect of DOC on Inflammation in Partially Hepatectomized Animals*

Female Sprague-Dawley rats weighing from 155–165 gm. were either partially hepatectomized (70%) or sham operated, under ether anesthesia. When the operations

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were completed, the animals were re-anesthesized with ether and granuloma pouches were made according to a technique previously described (3). It consists briefly of injecting 25 cc. of air under the shaved dorsal skin, followed by injection of 0.5 cc. of 1% croton oil in cottonseed oil. After two days, air was withdrawn and the animals were killed after 5 days, instead of the usual 4 day period, because the stress of the operations (sham and hepatectomy) retarded the rate of exudate formation. At autopsy, the volume of exudate was accurately measured in a graduated cylinder, and in two of the experiments reported here, the thymus was weighed in the fresh state. DOC was given at the total daily dose of 8 mg., divided into two morning and two afternoon injections of 2 mg. each. Volume of each injection was 0.2 cc. and the vehicle consisted of a solution of carboxymethylcellulose, polysorbate 80 and benzyl alcohol in isotonic saline. Injections were made in chest and abdomen, far from the pouch, in order to avoid any local effect.

Results. Results are summarized in Table 1. At the dose used, DOC was inactive in the sham operated animals whereas it was antiphlogistic, thymolytic and catabolic after hepatectomy.

TABLE 1. EFFECT OF DOC ON INFLAMMATION, THYMUS AND BODY WEIGHT IN PARTIALLY HEPATECTOMIZED ANIMALS

	I		II			III		IV		
	Sham					Hepatectomy				
	Vehicle		DOC		Significance	Vehicle		DOC		Significance
		S		S			S		S	
Daily dose			8 mg.					8 mg.		
No. of rats	12		9			12		10		
Body weight difference (gm.)	+2		+2			+3		−6		
Exudate (cc)	8.0	0.84	8.6	0.85	I–II N.S.	4.6	0.55	2.0	0.26	III–IV **
% Inhibition			−7					56		
Thymus (mg.)	314.6	17.9	291.2	10.0	I–II N.S.	256.6	18.8	147.9	10.7	III–IV ***
% Inhibition			S					42		

S: Standard error.
N.S.: Difference is not significant.
** "P" <0.01.
*** "P" <0.001.

2. *Is the Antiphlogistic Property of DOC Specific or Due to a Stress Reaction?*

Since desoxycorticosterone may elicit important metabolic derangements when given at high doses, it was necessary to verify whether the antiphlogistic effect observed was specific or the result of a stress reaction. To check this possibility, three experiments were performed in which adrenalectomized rats, force-fed animals and animals given supplements of potassium chloride to drink, were used, respectively. Female Sprague-Dawley rats weighting from 150–170 g. were used throughout.

A. *Antiphlogistic effect of DOC in adrenalectomized animals*

Andrenalectomy was performed under ether anesthesia through two low incisions, as anterior as possible—in order not to interfere with the granuloma pouch—whereas controls were sham operated. After surgery, granuloma pouches were prepared with a new ether anesthesia. The four day assay was used as described before (3). All animals were given saline 1% to drink throughout the experiment. DOC was administered at the total daily dose of 15 mg., divided into five injections of 3 mg. in 0.2 cc., subcutaneously.

TABLE 2. EFFECT OF DOC ON INFLAMMATION IN ADRENALECTOMIZED ANIMALS

	I		II			III		IV		
	Sham					Adrenalectomy				
	Vehicle		DOC		Significance	Vehicle		DOC		Significance
		S		S			S		S	
Daily dose			15 mg.					15 mg.		
No. of rats	10		10			9		10		
Body weight difference (gm.)	+1		−7			−2		+1		
Saline consumption (cc. per day)	36		65			26		75		
Food consumption (gm. per day)	13.5		12.0			10.8		13.1		
Exudate (cc.)	5.6	0.35	2.4	0.28	I–II ***	6.0	0.34	2.0	0.24	III–IV ***
										II–IV N.S.
% Inhibition			56					67		

S: Standard error.
N.S.: Difference is not significant.
*** "P" <0.001.

Results. As shown in Table 2, DOC exerted approximately the same antiphlogistic effect in adrenalectomized as in normal animals. As expected, the daily saline consumption was increased by DOC. In the sham operated animals (Group II), DOC was catabolic whereas it only slightly increased the body weight of the adrenalectomized rats (Group IV).

B. *Antiphlogistic effect of desoxycorticosterone in force-fed animals*

Not only does DOC inhibit inflammation, but at the same time growth is retarded. In order to determine whether this effect on body weight was merely due to a decrease in food intake, DOC was administered in force-fed rats. It was thought that this experiment would also indicate if the inhibition of inflammation could be attributed to a decrease in food consumption.

Forty rats were divided into four equal groups as shown in Table 3. The first two groups were given granulated Purina laboratory chow *ad lib.* and the amount consumed daily was measured. The last two groups were force-fed with 10 cc. of a medium carbohydrate diet twice daily and water was allowed throughout. It had been learned by previous experience that rats receiving this quantity of fluid diet grow as well as animals

TABLE 3. EFFECT OF DOC ON INFLAMMATION AND BODY WEIGHT IN ANIMALS FED AD LIB. AND FORCE-FED

	I		II			III		IV		
	Fed *ad lib.*					Force-fed				
	Vehicle		DOC		Significance	Vehicle		DOC		Significance
		S		S			S		S	
Daily dose			15 mg.					15 mg.		
No. of rats	10		10			10		10		
Body weight difference (gm.)	+9		−4			+11		+5		
Food consumption (gm. per day)	15.1		13.4							
Exudate (cc.)	6.8	0.60	2.8	0.29	I–II ***	6.9	0.48	3.3	0.36	III–IV ***
										II–IV N.S.
% Inhibition			58					44		

S: Standard error.
N.S.: Difference is not significant.
*** "P" <0.001.

TABLE 4. EFFECT OF DOC ON INFLAMMATION AND BODY WEIGHT IN ANIMALS GIVEN SUPPLEMENTS OF POTASSIUM

	I		II			III		IV		
	Water					KCl 2%				
	Vehicle		DOC		Significance	Vehicle		DOC		Significance
		S		S			S		S	
Daily dose			15 mg.					15 mg.		
No. of rats	10		10			10		10		
Body weight difference (gm.)	+8		0			+10		+2		
Fluid consumption (cc. per day)	27		32			28		29		
Food consumption (gm. per day)	14.7		13.9			15.2		13.8		
Exudate (cc.)	6.6	0.54	4.1	0.30	I–II **	6.5	0.35	3.8	0.49	III–IV ***
										II–IV N.S.
% Inhibition			37					41		

S: Standard error.
N.S.: Difference is not significant.
** "P" <0.01.
*** "P" <0.001.

eating Purina *ad lib.* These two food preparations were given for a training period of 5 days after which granuloma pouches were made. The animals were killed after 4 days and the exudate collected. DOC was administered at a daily dose of 15 mg., divided into 5 subcutaneous injections of 3 mg. (two A.M., three P.M.).

Results. DOC treated animals of Group II ate on the average 1.7 gm. less daily than controls and lost weight (Table 3). In the force-fed DOC treated rats (Group IV), there was no weight loss but the gain was small compared with the controls (Group III). This suggests that only part of the catabolic effect of DOC is due to decreased appetite. Whether the animals ate *ad lib.* or were force-fed, DOC exerted its usual antiphlogistic effect and the difference between the two DOC treated groups was not statistically significant.

C. *Antiphlogistic effect of desoxycorticosterone in animals drinking KCl 2%*

Forty animals were divided into 4 equal groups as indicated in Table 4. The first two groups drank water and the last two were given a 2% solution of KCl to drink. If the kypokalemic effect of DOC was so great that it provoked a stress reaction in the animals, it was hoped that this supplement of KCl would prevent this complication. The animals were observed for 4 days, during which fluid intake was measured daily; it was found that about as much of the KCl solution at this concentration was consumed as water. After this period, granuloma pouches were made and the animals of the last two groups continued receiving KCl 2%. DOC was injected at a total daily dose of 15 mg., divided into 5 subcutaneous injections of 3 mg. (two A.M., three P.M.). All rats were given granulated Purina laboratory chow *ad lib.* The animals were killed after 4 days and the exudate collected.

Results. Table 4 indicates that the percentage of inhibition of inflammation was the same whether the animals drank water or KCl 2%. Likewise, KCl did not influence the catabolic effect of DOC. Daily food consumption was diminished by about 1 g. in both groups receiving the steroid. A daily fluid intake of 30 cc. (Table 4) indicates that the animals received a supplement of 600 mg. of KCl (314 mg. of potassium) daily.

DISCUSSION

The results of the first experiment indicate that removal of 70% of the liver increases the antiphlogistic, thymolytic and catabolic properties of desoxycorticosterone. The apparent ease with which the liver handles this steroid may partially explain why high doses have to be administered systemically, in intact animals, in order to produce the effects described.

The last three experiments showed to our satisfaction that the antiphlogistic effect of DOC is not due to a pituitary-adrenal stress reaction elicited by metabolic changes produced by the treatment. Actually, the degree of inhibition of inflammation was practically the same whether the animals were adrenalectomized, force-fed or given supplements of KCl. We therefore feel justified to conclude that the antiphlogistic, thymolytic and catabolic activities of DOC are specific. They seem to be as specific as in the case of glucocorticoids, the only difference between the two classes of compounds being in degree. The parallelism is even more striking in view of the results of Verzár (4) who demonstrated that DOC promotes liver glycogen deposition in the adrenalectomized rat. However, the possibility that DOC exerts its cortisol-like activity through systemic, extra-adrenal stressing effect must be kept in mind since it is not ruled out by our experimental conditions. Such a possibility only appears to be remote as judged by the excellent clinical condition of the DOC treated animals.

As to the mechanism by which DOC and other 11-desoxysteroids (2) exert their antiphlogistic, catabolic and thymolytic actions, it is possible that either a) they are active as such but are weak compounds compared to steroids of the cortisone family; or b) they may exert these activities only after conversion into another compound, probably steroidal. This hypothetical conversion product would be the active principle and the conversion would take place in organs other than the adrenals and the liver.

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ANTIPHLOGISTIC PROPERTY OF CERTAIN 11-DESOXY STEROIDS AFTER SYSTEMIC ADMINISTRATION

ANDRÉ ROBERT AND JAMES E. NEZAMIS

Department of Endocrinology, The Upjohn Co., Kalamazoo, Michigan

ABSTRACT

Two 11-desoxy corticoids, desoxycorticosterone (DOC) and Reichstein's Cpd. "S," were found to be antiphlogistic in the rat (using the granuloma pouch), after either systemic or local administration. In addition, when given systemically in large doses, they were catabolic and thymolytic. These findings assimilate these steroids to glucocorticoids although they have generally been classified among mineralocorticoids. Progesterone, given systemically, was slightly antiphlogistic and thymolytic. Its antiphlogistic property was more evident after local administration.

The following hypotheses are considered to explain the mode of action of these 11-desoxy steroids on inflammation, thymus and body weights:

1. These steroids may be active as such but, being weak or rapidly inactivated by the liver, large doses must be administered.

2. These steroids may exert their activity only after being converted in the body into another compound (possibly the correspondent 11-oxy derivatives) endowed with the described properties.

3. Since large doses have to be given, these steroids may produce such metabolic disturbances that the antiphlogistic effect observed would be due to non-specific stress reaction.

The data do not permit a choice among these hypotheses.

DESOXYCORTICOSTERONE (DOC), administered systemically, has repeatedly been reported to exert a prophlogistic effect in experimental animals (1–7). Seifter *et al.* (8) found that DCA produces a marked increase in synovial membrane permeability in the rabbit, by using the clearance rate of phenolsulfonphthalein from the joint, whereas cortisone exerts the opposite effect. There were, however, some reports several years ago on the alleged anti-arthritic effect of DOC associated with ascorbic acid (9–11), but the majority of clinicians who attempted to reproduce these latter results failed; they found this steroid either to be ineffective (12–15) or to aggravate the inflammatory process (16–18). In another series, Cajander (19) found this hormone to be of no value in some patients, anti-arthritic in others, and prophlogistic in still other cases. Finally, in some cases of Addison's disease, treatment with DCA has been followed by development of "rheumatic arthritis" which subsided upon administration of cortisone (20, 21).

On the other hand, we found that this same steroid, when locally applied

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into an inflammatory area, inhibits inflammation (22). It was then assumed that, after local administration, the tissue concentration of the injected material is much higher than following systemic administration; for this reason, it was postulated that, to demonstrate an antiphlogistic effect for DOC after systemic administration, one would have to give large doses to build up a concentration at the tissue level (the inflamed area) high enough to inhibit inflammation. To check this hypothesis, large doses of desoxycorticosterone were administered subcutaneously to granuloma pouch bearing animals. Other 11-desoxy steroids were also used for comparison purposes.

MATERIALS AND METHODS

Female Sprague-Dawley rats, weighing from 155–165 gm., were used, each group consisting of 8 to 15 animals. Granuloma pouches were performed according to a technique previously described (23). In brief, it consisted of injecting 25 cc. of air under the shaved dorsal skin, followed by injection into the air sac of 0.5 cc. of 1% croton oil in cottonseed oil. Two days after the pouch was made, air was withdrawn and the animals were killed on the fourth day. The exudate was collected in a graduated cylinder and, in some experiments, the pouch itself was dissected out and weighed after being emptied of its contents. The hormonal treatment was initiated immediately after the pouch was made. Desoxycorticosterone (DOC), Reichstein's compound "S" (Cpd. "S"), progesterone and pregnane-3,20-dione were given in various amounts and the total daily dose was divided into several subcutaneous injections of 0.2 cc., as indicated in Table 1. Injections were made over the chest and abdomen, that is, far from the granuloma pouch. Control animals received the same number of injections of vehicle (a solution of carboxymethylcellulose, polysorbate 80 and benzyl alcohol in isotonic saline).

In the case of local administration, the steroids were first injected under the dorsal skin in a volume of 0.2 cc., followed by injection of air about 20 minutes later; all the steroid material was thus lifted and remained in the wall of the granuloma pouch throughout the experiment. No further injection was made in these animals.

RESULTS

Figure 1 indicates that DOC and Cpd. "S" are antiphlogistic when administered systemically; the minimal effective doses are around 7 mg. and 20 mg. daily, respectively, and the degree of inhibition of inflammation is related to the dose. In the case of progesterone, a dose response curve could not be established satisfactorily because activity was found for only the highest dose; the threshold dose may be around 70 mg. a day. Perusal of Table 1 shows that when given at effective antiphlogistic doses, both DOC and Cpd. "S" are also catabolic, as shown by body weight changes, and thymolytic. By comparing the doses of both steroids required to obtain a given percentage of inhibition of inflammation, DOC appears to be two to three times as active as Cpd. "S." DOC is also more potent than Cpd. "S" as a catabolic and thymolytic agent, whereas progesterone is not catabolic and its antiphlogistic and thymolytic effects are slight.

Furthermore, Table 1 indicates that these three steroids are also anti-

TABLE 1. EFFECT OF DOC, CPD. "S" AND PROGESTERONE ON INFLAMMATION, THYMUS AND BODY WEIGHT

Treatment	Daily Dose	No. rats	Body weight change (g.)	Exudate (cc.)			Granuloma pouch (mg.)			Thymus (mg.)		
					S	Significance		S	Significance		S	Significance
Systemic administration												
No Treatment		10	+ 8	6.1	0.37		1987	95.7		340	18.8	
Vehicle	0.2 cc.×5	10	+ 5	6.9	0.54	N.S.	2223	189.7	N.S.	316	19.0	N.S.
Vehicle	0.2 cc.×5	15	+ 7	6.8	0.39		2313	91.6		378	20.5	
Pregnane-3,20-dione	15 mg. (5 mg.×3)	15	+11	6.0	0.36	N.S.	2189	79.9	N.S.	374	13.5	N.S.
Vehicle	0.2 cc.×5	10	+ 6	7.4	0.47		2598	164.2		315	15.6	
Desoxycorticosterone	2.5 mg. (0.5 mg.×5)	10	+ 8	7.9	0.65	N.S.						
	5 mg. (1 mg.×5)	10	+ 3	7.3	0.60	N.S.						
	10 mg. (2 mg.×5)	10	− 3	4.5	0.62	**						
	15 mg. (3 mg.×5)	9	− 7	2.4	0.37	***						
	20 mg. (4 mg.×5)	10	− 8	1.6	0.26	***						
	25 mg. (5 mg.×5)	9	−11	1.4	0.23	***	1204	58.6	***	120	7.2	***
Vehicle	0.2 cc.×5	10	+ 6	7.3	0.47							
Reichstein's Cpd. "S"	10 mg. (5 mg.×2)	10	0	7.5	0.50	N.S.						
	15 mg. (5 mg.×3)	10	+ 2	7.2	0.61	N.S.						
	20 mg. (5 mg.×4)	10	+ 1	5.0	0.70	*						
	25 mg. (5 mg.×5)	10	− 2	5.2	0.59	*						
	30 mg. (5 mg.×6)	10	− 4	3.8	0.74	**						
Vehicle	0.2 cc.×5	10	+11	6.1	0.32		2065	154.9		414	17.5	
Reichstein's Cpd. "S"	40 mg. (5 mg.×8)	10	− 3	2.2	0.62	***	1314	121.9	***	235	16.6	***
Vehicle	0.2 cc.×10	10	+ 4	6.5	0.67							
Reichstein's Cpd. "S"	50 mg. (5 mg.×10)	10	− 8	2.2	0.46	***						
Vehicle	0.2 cc.×5	8	+ 3	6.5	0.34							
Progesterone	25 mg. (5 mg.×5)	8	+ 8	7.1	0.50	N.S.						
Vehicle	0.2 cc.×5	10	+11	6.1	0.32							
Progesterone	50 mg. (5 mg.×10)	10	+ 8	5.3	0.33	N.S.						
Vehicle	0.2 cc.×20	10	+10	5.4	0.33		1944	92.5		364	16.9	
Progesterone	100 mg. (5 mg.×20)	10	+ 4	3.4	0.28	***	1532	76.6	**	270	21.3	**
Local administration												
No Treatment		10	+ 8	6.1	0.37		1987	95.7		340	18.8	
Vehicle	0.2 cc.	10	+ 7	6.4	0.41	N.S.	1857	88.3	N.S.	341	28.7	N.S.
Vehicle	0.2 cc.	10	+ 4	6.1	0.46							
Pregnane-3,20-dione	15 mg.	10	+ 3	6.0	0.27	N.S.						
Vehicle	0.2 cc.	8	+10	6.5	0.58							
Desoxycorticosterone	10 mg.	10	+ 6	1.3	0.36	***						
Vehicle	0.2 cc.	10	+ 7	5.9	0.63							
Reichstein's Cpd. "S"	10 mg.	10	+ 5	1.6	0.26	***						
Vehicle	0.2 cc.	10	+ 5	7.7	0.35							
Progesterone	10 mg.	10	+10	2.5	0.66	***						

S: Standard error.
N.S.: Difference is not significant.
* "P"<0.05.
** "P"<0.01.
*** "P"<0.001.

phlogistic when administered locally, but their relative difference in potency is by far not as marked as after systemic administration. In another study (unpublished) in which the local effect of multiple doses was examined, it was found that DOC and Cpd. "S" are about equally active and are 4 times as potent as progesterone.

It is noteworthy that these 11-desoxy steroids inhibited not only exudation but also the growth of inflammatory tissue as shown by the weight of the granuloma pouch. This indicates again that measure of exudate is as good an index of inflammation as weight of granuloma itself, a fact which has been repeatedly established in the past (24, 25). Finally,

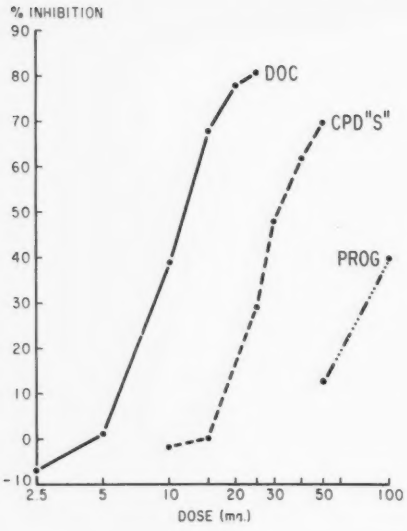

FIG. 1. Effect of DOC, Cpd. "S" and progesterone on inflammation. Ordinate indicates percentage of inhibition of exudate formation. Abscissa indicates dose per rat per day.

pregnane-3,20-dione, chosen as a representative of steroids lacking any known hormonal activity, was inactive.

DISCUSSION

The results of these experiments indicate that certain 11-desoxy steroids, when administered systemically, produce the same pharmacological changes (catabolism, inhibition of inflammation, thymus atrophy) as 11-oxy corticoids, provided high enough doses are given. DOC appeared to be the most active of the compounds studied, followed by Cpd. "S" (half to one-third as active as DOC) and progesterone (still less active). The effect obtained was directly related to the dose. Pregnane-3,20-dione, known to possess no hormonal activity, was inert. Previous lack of recognition of these properties may be ascribed to the fact that these steroids have rarely been administered systemically at the dose levels used in the present study.

In view of these observations, it is tempting to classify both DOC and Cpd. "S" among glucocorticoids, although they are commonly regarded as mineralocorticoids. It seems that they possess both activities, but at different degrees depending on the dose. This duality was known for glucocorticoids (e.g., cortisone, cortisol, and some of their derivatives, in addition to being antiphlogistic, catabolic and thymolytic, are also salt-retainers to various degrees). As far as we know, the reverse has never been firmly established, DOC being listed by many as a "pure" mineralocorticoid. There is no doubt that the classification of corticoids into *gluco* and *mineralo* is still most useful since it indicates the predominant bio-

logical activity of the steroids. These terms should not, however, be interpreted as suggesting exclusive properties. Multiple potentials often exist in a given steroid. Thus, DOC, like some new cortisol derivatives such as 2α-methyl-9α-fluorocortisol (26), may be classified as and act as a mineralocorticoid at very low doses but its inherent glucocorticoid properties appear only at very high doses.

Verzár and his co-workers have presented evidence for the glycogenic property of DOC. They found that DOC has the ability to deposit glycogen in the liver of adrenalectomized rats as cortisone does (27, 28). Both steroids appeared to differ only in the time required to produce the effect: a single dose of cortisone increased liver glycogen in a few hours, whereas several days of pretreatment were necessary to obtain the same change with DOC.

The literature concerning the local effect of 11-desoxy steroids on inflammation is rather confusing. Meier *et al.* (29) and Desaulles *et al.* (30) observed inhibition of granuloma formation after local application of desoxycorticosterone in subcutaneously implanted cotton pads. Similarly, Baker (31) reported a suppression of connective tissue surrounding a desoxycorticosterone pellet implanted into orbital tissue of the rat. Jasmin (32) confirmed this finding by studying the reaction around a DCA pellet implanted under dorsal skin. Other authors, however (Dougherty and Schneebeli (33), and Sakabe *et al.* (34) using respectively the spreading of loose connective tissue after histamine injection and the reaction of subcutaneously injected quartz), did not observe any local effect of DOC. Dougherty and Schneebeli (35) found that DOC counteracted the inhibitory property of cortisone. Taubenhaus *et al.* (36) could not demonstrate a local effect of DCA on the turpentine abscess, although when DCA and cortisone were mixed with turpentine, the inhibitory effect of cortisone was prevented by DCA. On the other hand, Rindani (37) reported a prophlogistic effect after injection of either DOC or Reichstein's compound "S" locally into the cavity of the granuloma pouch. Using Cpd. "S" locally, Dougherty and Schneebelli (33) found some anti-inflammatory activity. Progesterone injected together with quartz particles under the skin of the rat did not inhibit the resulting inflammation (34). Similarly, Opsahl (38) could not inhibit the spreading of India ink.

It is difficult to reconcile some of these conflicting reports. It is possible that steroids gave variable results because of the widely differing dosages used by these authors. Also, by using a variety of assays (turpentine abscess, quartz granuloma, tissue reaction to histamine, spreading of India ink, etc.), the response to a given steroid may not always be the same.

It is still difficult at this time to offer a good explanation as to the mode of action of these three 11-desoxy steroids, after either local or systemic administration. The following hypotheses may be considered.

A. These 11-desoxy steroids may be antiphlogistic as such when given

locally but may be so weak that, after systemic administration, the activity is not shown unless high doses are given; body dilution would prevent a high enough peripheral concentration to be reached. It is also possible that the steroids are so efficiently metabolized by the liver that only high doses can overcome such inactivation.

B. The steroids used may be inert but undergo conversion in the body after injection. The conversion products would then be endowed with antiphlogistic properties. One could speculate on the nature of these hypothetical compounds but, if they are steroidal in nature, they may be oxygenated at C-11. The 11-oxy derivatives of DOC, Cpd. "S" and progesterone are known to exert antiphlogistic activity by either local or systemic administration.

C. Finally, it could be argued that the high systemic doses used in our experiments induced such metabolic derangements that the antiphlogistic effect observed was due to non-specific stress reaction.

The experiments reported here do not permit choosing among these hypotheses but some of these were tested and are reported in a separate communication (39).

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EFFECT OF VARIOUS STEROIDS ON THE DEVELOPMENT OF THE BURSA FABRICII IN CHICK EMBRYOS[1]

RICHARD L. ASPINALL, ROLAND K. MEYER AND M. APPASWAMI RAO

Department of Zoology, University of Wisconsin, Madison, Wisconsin

ABSTRACT

The effects of various androgen-like steroids on the development of the bursa of Fabricius, the thymus and spleen were studied in chick embryos. Varying doses of the hormones, ranging from 0.05 mg. to 1.5 mg., were injected in oil solution into the albumin portion of developing eggs on the 5th day of incubation. Autopsies were performed on the 19th day of incubation.

Of the compounds tested, dihydrotestosterone proved to be the most active in terms of inhibiting the development of the bursa, using the degree of reduction of the tissue weight as the criterion of activity. Several of the other less potent compounds were grouped in the order of decreasing activity as follows: a) androsterone, androstane-3,17-dione, b) 19-nortestosterone, testosterone-cyclo-pentyl-propionate, methylandrostenediol, and c) testosterone.

An attempt was made to relate the relative activities of these compounds in causing bursal inhibition to their structural configurations. For example, those androgens with a completely reduced steroid nucleus were shown to be the most active.

The development of the thymus and spleen was not affected by any of the compounds at the doses employed.

MEYER *et al.* have shown that in chickens (1, 2, 3) and pheasants (2) 19-nortestosterone inhibits the embyonic development of the bursa Fabricii, a lymphoid organ attached to the dorsal wall of the cloaca in birds. A detailed histological investigation of this phenomenon revealed that under the influence of the hormone the bursal anlage is arrested at the endodermal duct stage (4). Differentiation into lymphoid tissue does not take place.

On the basis of these data it became of interest to make a comparative study of the effects of other androgenic steroids on the differentiation of the bursa. This report describes the results of such a study.[2]

MATERIALS AND METHODS

Eggs from a hybrid strain of the White Leghorn chicken known as the "Wisco-white" (Sunnyside Hatchery, Madison, Wisconsin) were selected for this study. They were

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maintained in an incubator at a temperature of 99½° F and a relative humidity of 85%. The eggs were removed from the incubator long enough for treatment on the 5th day of incubation. After candling, a hole was made with a sharp instrument at the pointed end of the fertile egg. The hormone solutions were then injected into the albumin with a ½ inch 23 gauge hypodermic needle attached to a 1 cc. syringe. All hormones were injected in 0.1 cc. volume of corn oil (U.S.P. Magnus, Mabee and Reynard, Inc., New York, N. Y.). The hole was covered with melted paraffin and the eggs were immediately returned to the incubator.

The embryos were autopsied on the 19th day of incubation, 2 days prior to hatching. The eggs were first placed in a chloroform chamber for a few minutes to kill the embryos. The shell, shell membranes, and extraembryonic membranes, with the exception of the yolk sac, were removed. The embryos were wiped with absorbent cotton and weighed on an O'Haus triple-beam balance. The left thymus, the spleen and bursa were then dissected free of extraneous tissue and weighed on a 100 mg. Roller-Smith balance.

The data were calculated in terms of mg. of tissue per 100 gm. body weight and expressed as mg.%.

RESULTS

Each compound was tested at 2 dose levels in the first experiment to establish its activity relative to 19-nortestosterone. We had previously established that 0.63 mg. 19-nortestosterone was sufficient to completely inhibit the development of the bursa (1, 2, 3, 4). Therefore, the doses of this hormone were reduced to 0.1 mg. and 0.3 mg. to attain a dose-response relationship. The other steroids were tested at 0.5 mg. and 1.5 mg. to ascertain which compounds were as active and which were less active than 19-nortestosterone. The steroids tested differed from each other in the side groups on carbons 3 and 17 and in the saturation of rings A and B. This design was selected with the intent that the bursal inhibitory activity might be correlated with specific molecular configurations.

The results of the first experiment (Table 1) revealed that androstane-3,17-dione, methylandrostenediol, androsterone and dihydrotestosterone were at least as active as 19-nortestosterone in inhibiting the development of the bursa. Dehydroepiandrosterone, androstane-17-one, Δ^4-androstene-3,17-dione and estradiol-17-β, in the order of decreasing activity, were decidedly weaker at these doses. Δ^5-androstane-3,17-diol-17-benzoate and 17-hydroxypregnenolone showed no activity when compared to control values. The thymus and spleen were not significantly affected by any of the compounds.

In the next experiment the most active compounds were given at lower doses to determine their relative activities in causing inhibition (Table 2). Dihydrotestosterone was considered to be the most active when judged by its lower extinction value, the smallest dose of a compound which produces maximum inhibition. The extinction value for dihydrotestosterone is assumed to be 15.7 mg.% at the 0.05 mg. dose level. Androsterone and androstane-3,17-dione had similar activities, possessing somewhat higher extinction values (approximately 35 mg.% at the 0.1 mg. dose) than di-

TABLE 1. EFFECT OF VARIOUS STEROIDS ON THE DEVELOPMENT OF THE THYMUS, SPLEEN AND BURSA OF FABRICIUS IN THE EMBRYOS OF WISCO-WHITE CHICKENS

Dose		0.5 mg.			1.5 mg.		
Treatment	Group	Mg.% thymus* S.E.	**Mg. % spleen S.E.	Mg. % bursa S.E.	Mg.% thymus* S.E.	Mg. % spleen S.E.	Mg. % bursa S.E.
Androsterone	2	53.7±6.6 (12)	31.9±1.7 (12)	0.0±0.0 (12)	59.9±3.2 (12)	29.9±1.4 (11)	0.0±0.0 (12)
Δ^5-androstane-3-17-diol-benzoate	3	64.6±7.1 (12)	29.6±2.3 (12)	119.6±7.6 (12)	52.6±5.1 (11)	23.7±2.7 (12)	92.4±5.0 (12)
Androstane-3,17-dione	4	64.4±5.8 (11)	27.2±2.3 (11)	0.2±0.1 (11)	63.9±3.9 (11)	30.2±1.7 (11)	0.0±0.0 (11)
Dehydroepiandrosterone	5	59.7±6.6 (12)	32.3±3.3 (12)	42.7±6.2 (12)	54.5±3.9 (12)	27.8±2.9 (12)	0.0±0.0 (12)
Androstane-17-one	6	65.9±7.5 (11)	26.5±2.3 (11)	52.2±8.7 (11)	60.7±5.1 (11)	24.4±1.5 (11)	20.8±5.2 (11)
Methylandrostenediol	7	58.5±5.4 (9)	24.0±1.6 (9)	3.6±1.9 (9)	60.6±7.1 (10)	27.1±2.6 (10)	2.4±1.2 (9)
Δ^4-androstene-3,17-dione	8	59.2±3.9 (10)	28.4±1.6 (10)	73.7±6.1 (10)	57.8±3.8 (11)	23.9±1.8 (11)	21.5±4.0 (11)
17-hydroxypregnenolone	9	65.7±4.4 (11)	30.2±1.4 (11)	107.0±5.7 (11)	63.6±7.5 (10)	25.8±1.1 (10)	118.4±6.3 (10)
α-estradiol	10	57.8±4.3 (12)	27.2±1.1 (12)	80.6±6.3 (12)	56.2±4.4 (12)	32.4±1.4 (12)	57.8±5.1 (12)
Dihydrotestosterone	11	55.6±5.7 (12)	23.4±1.9 (12)	2.2±1.4 (12)	57.3±5.4 (12)	31.7±1.9 (12)	1.2±0.8 (12)
		0.1 mg.			0.3 mg.		
19-nortestosterone	12	55.2±3.7 (12)	26.3±1.3 (12)	55.6±6.4 (12)	56.1±3.4 (11)	25.7±1.6 (11)	10.5±2.9 (11)
		Oil only			No treatment		
Controls	1	66.2±6.1 (10)	31.6±3.1 (11)	95.9±7.2 (11)	73.6±6.7 (12)	28.5±1.5 (12)	112.6±7.8 (12)

* The anterior 3 lobes of the left thymus.
** Mg. tissue per 100 gm. body weight ± standard error.
The number in parentheses under each value represents the number of embryos.

hydrotestosterone. The 0.2 mg. value for androstane-3,17-dione had a very large standard error, which is unexplainable (Table 2). The true value may be somewhat lower.

Methylandrostenediol, 19-nortestosterone and testosterone-cyclo-pentyl-propionate gave curves falling within the same range with similar extinction values. They, therefore, appear to be equal in activity, but somewhat less active than androsterone and androstane-3,17-dione in causing bursal inhibition.

19-nortestosterone had more activity when applied to the shell in oil solution than when injected into the albumin. When applied in this manner the thymus and spleen were also substantially reduced in size (Table 2).

DISCUSSION

On the basis of the quantitative data obtained, the relative potency of the most potent steroids in causing bursal inhibition can be arranged in terms of decreasing activity as follows: 1) dihydrotestosterone, 2) androsterone, androstane-3,17-dione, 3) 19-nortestosterone, methylandrostenediol, testosterone-cyclo-pentyl-propionate, and 4) testosterone.

TABLE 2. EFFECT OF VARIOUS STEROIDS ON THE DEVELOPMENT OF THE THYMUS, SPLEEN AND BURSA OF FABRICIUS IN THE EMBRYOS OF WISCO-WHITE CHICKENS

Dose	0.05 mg.	0.1 mg.	0.2 mg.	0.4 mg.	0.05 mg.	0.1 mg.	0.2 mg.	0.4 mg.	0.05 mg.	0.1 mg.	0.2 mg.	0.4 mg.
Treatment	Mg.% bursa +S.E.*				Mg.% Left thymus +S.E.**				Mg.% Spleen ±S.E.			
19-nortestosterone (injected)	—	56.9±3.9 (10)	41.8± 5.2 (11)	3.3± 1.8 (12)	—	94.4± 6.1 (9)	74.1±5.2 (9)	86.3±5.0 (9)	—	32.3±2.9 (9)	26.4±2.4 (9)	30.2±2.1 (9)
19-nortestosterone (on shell)	—	11.6±2.4 (10)	3.7± 2.0 (12)	0.1± 0.0 (9)	—	47.4± 3.2 (9)	47.3±3.5 (9)	56.6±5.0 (9)	—	25.1±2.8 (9)	19.2±1.5 (9)	22.4±1.5 (9)
Testosterone-cyclopentyl-propionate	—	65.0±8.6 (10)	31.4± 6.3 (12)	7.2± 2.7 (11)	—	81.8± 6.2 (9)	88.6±5.0 (9)	75.9±4.5 (9)	—	31.9±3.4 (9)	34.7±4.8 (9)	32.3±5.5 (9)
Testosterone	—	77.4±6.9 (11)	64.9± 9.9 (9)	66.7±10.0 (9)	—	83.5±10.6 (9)	80.2±3.9 (9)	67.9±2.9 (9)	—	27.2±1.6 (9)	24.5±1.6 (9)	25.4±1.4 (9)
Androsterone	74.3±9.9 (9)	35.5±6.3 (9)	11.0± 3.8 (12)	—	87.6±7.4 (9)	75.8± 3.0 (9)	78.0±5.2 (9)	—	30.8±2.6 (9)	29.5±2.4 (9)	29.5±1.0 (9)	—
Androstane-3,17-dione	44.3±5.3 (11)	29.7±4.8 (12)	35.5±13.1 (12)	—	79.7±4.3 (9)	81.1± 9.7 (9)	77.6±6.7 (9)	—	30.7±3.7 (9)	28.5±1.9 (9)	32.0±3.3 (9)	—
Methylandrostenediol	71.5±9.5 (12)	67.4±7.4 (10)	31.1± 5.3 (9)	—	86.1±8.4 (9)	88.0± 3.7 (9)	86.1±3.1 (9)	—	28.6±3.6 (9)	32.0±1.9 (9)	30.3±2.1 (9)	—
Dihydrotestosterone	15.7±3.4 (12)	7.2±2.6 (11)	1.3± 0.2 (11)	—	100.9±4.9 (9)	97.4± 7.3 (9)	99.7±6.7 (9)	—	28.9±1.8 (9)	29.4±2.2 (9)	36.5±4.0 (9)	—

	Mg.% Bursa ±S.E.	Mg.% Left thymus ±S.E.	Mg.% Spleen ±S.E.
Controls (corn oil)	116.5±6.5 (12)	101.2±7.9 (9)	26.1±2.4 (9)
Controls (no treatment)	103.9±6.5 (11)	77.4±5.7 (9)	30.1±2.3 (9)

* Mg. tissue per 100 gm. body weight ± standard error.
** Includes the entire left thymus.
The number in parentheses under each value represents the number of embryos.

It became of interest to correlate relative bursal inhibitory capacity with the molecular structure of the androgenic steroids studied. We are fully aware, however, that the interpretations derived can only be tentative, because of the small number of compounds tested. Dihydrotestosterone, the most potent of the compounds, has the following characteristics: a) 3-keto group, b) fully saturated steroid nucleus, c) 17-hydroxyl group, and d) 18 and 19 methyl groups. Androstane-3,17-dione has the same characteristics, except a 17-keto group replaces the 17-hydroxyl group. Androsterone, likewise, has a 3-alpha-hydroxyl group instead of a 3-keto group. Since these compounds are less active, the 3-keto and 17-hydroxyl configurations seem to be important.

The compounds in the next group, which include methylandrostenediol, 19-nortestosterone and testosterone-cyclo-pentyl-propionate, all possess an unsaturated steroid nucleus. This is also true of the remainder of the compounds with the exception of androstane-17-one. This fact suggests that a reduction of the steroid nucleus leads to greater activity. A comparison of the structures and activities of androstane-3,17-dione and Δ^4-androstene-3,17-dione and of androsterone and dehydroepiandrosterone bears this out (Tables 1, 2). Jedeikin and White (5), however, state that reduction of ring A did not alter glucose oxidation, oxygen consumption and protein and nucleic acid synthesis of lymphocytes *in vitro* and *in vivo*.

Testosterone and 19-nortestosterone differ from one another only by the absence of the 19-methyl group in the latter, which is associated with an increase in activity. Similar results were obtained by White (6) when these steroids were added *in vitro* to thymic lymphocyte suspensions. A greater inhibition of endogenous respiration, oxygen consumption and glucose oxidation was obtained with 19-nortestosterone.

Preliminary results using Nilevar (17-ethyl-19-nortestosterone) indicate that it is considerably more potent than dihydrotestosterone as a bursal inhibitor. Fifty μg Nilevar resulted in complete bursal inhibition and 1 μg was sufficient to reduce it approximately 50%. It is interesting to note that Edgren *et al.* (7) found estrogen antagonistic potency to be enhanced by an ethyl group at position 17 of 19-nortestosterone. The 17-ethyl group thus tends to augment the activity of the compound as an estrogen antagonist and as a bursal inhibitor.

Perhaps the bursal inhibitory potency of these steroids depends upon their metabolism in the embryo. The degree of activity may be dependent upon the relative amount of each steroid that is metabolized to a more potent analogue.

The reason for the greater activity of 19-nortestosterone when applied to the shell is not known. One assumption, which seems reasonable, is that when the hormone is injected it remains in one or several drops in the albumin, thus limiting the contact between albumin and hormone to a relatively small surface. When applied to the shell the contact area

would be greater and more hormone could thus become available to the embryo.

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RENAL EXCRETION OF PROTEIN IN ADRENALECTOMIZED DOGS TREATED WITH VARIOUS ADRENAL CORTICAL HORMONES[1]

ROBERT D. COYE AND JAMES DONKLE

Department of Pathology, University of Wisconsin Medical School, Madison, Wisconsin

ABSTRACT

The excretion of urinary protein was studied in four adrenalectomized dogs given different types and levels of adrenal cortical hormone replacement. Distinctive changes in the levels of serum proteins as well as changes in serum electrolytes were associated with only minor alterations in the rate of urine protein excretion. An increase in the clearance of globulins during the period of high cortisone dosage was noted. This was due to diminished serum concentrations of this fraction while the excretion rate remained constant. These data suggest that the adrenal cortical hormones play a minor role in the regulation of renal protein excretion by the normal dog.

THE rate of excretion of protein by the kidney is influenced in certain instances by the level of the adrenal hormones. Addis (1) demonstrated that adrenalectomy reduced the proteinuria in normal rats, and that the proteinuria was restored to its previous level by treatment with cortical hormones. Similarly, the proteinuria resulting from renin administration in rats could be abolished by adrenalectomy and restored with ACE, DCA, or Compound E. ACTH has been shown to augment both the normal protein excretion by the rat and also the proteinuria following renin administration (2). Adrenalectomized rats given nephrotoxic serum excrete significantly less protein than their non-adrenalectomized controls (3). These experiments suggest a permissive effect of the hormone in rats. The association of "stress" situations such as fever with increased urine protein excretion is well known, and suggests a possible adrenal relationship. The successful use of adrenal cortical hormones in the nephrotic syndrome is evidence of an opposite effect of these hormones on the excretion of urine protein. The nature of the effect of these hormones is not understood, and whether it is of significance in animals other than rats which normally exhibit a degree of proteinuria as well as sex variation in protein excretion not seen in other mammals is open to question. Alterations of the serum protein levels such as the elevation of the serum alpha globulin following cortisone administration (4) might be of importance.

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The present study was designed to assess the possible effect of adrenal cortical hormones on urine protein excretion in dogs.

MATERIALS AND METHODS

Four adult male mongrel dogs weighing from 13 to 15 kg. were adrenalectomized as follows. Food and water were withheld 12 hours before operation. The dogs were anesthetized with oxygen and ether after premedication with 15 mg./kg. of nembutal and 0.4 mg. atropine sulphate. A generous midline laparatomy incision provided adequate exposure of the adrenals which were resected by blunt and sharp dissection. Following resection of the left gland, 5.0 mg. of DCA and 50 mg. of hydrocortisone were administered, and following resection of the right gland 125 ml. of warm saline with 5% dextrose and 500,000 u. of penicillin were administered. The dogs were given 5 mg. DCA and from 20 to 30 mg. of hydrocortisone per day for the first five days post-operative. From the 5th to the 15th post-operative days they received no cortisone and the dose of DCA was gradually reduced to 1–1.5 mg./day. A period of several days with no replacement drug produced obvious signs of adrenal cortical insufficiency including weakness, anorexia and high serum potassium values. Following the withdrawal period the dogs were maintained on 1–1.5 mg. DCA/day and appeared strong and healthy. The diet consisted of standard kennel rations and tap water *ad lib.* The experimental periods began 26–59 days following adrenalectomy, at which time the operative wounds were completely healed. The dogs were studied in pairs during successive 35 day periods as follows: 7 days of DCA 1.0 mg./day, 7 days with no replacement drug, receiving only 500–1000 ml. of intraperitoneal salt solution as described by Grollman (5), 4 days of cortisone 2.5 mg./kg./day, 5 days of cortisone 10 mg./kg./day, and 12 days on the control DCA dosage of 1.0 mg./day. During these periods, the following measurements were made: body weight, rectal temperature, 24 hour urine volume, urine specific gravity, total urine and serum protein, serum potassium and sodium, and hematocrit. Total serum protein was determined by the micro-Kjeldahl method. Aliquots of the 24 hour urine collections were filtered, dialysed against running tap water, lyophilized and the resulting powder used for total urine protein determination by the Lowry method (6) and for fractionation by filter paper electrophoresis in a Durrum type cell. The strips were stained with brom phenol blue and scanned with a Spinco Analytrol. Serum proteins were fractionated by the same technique. Serum protein clearances were calculated by dividing the 24 hour urine protein value by the serum concentration of the corresponding electrophoretic fraction.

RESULTS

The effects of the various substances are summarized for each dog in Table 1.

DCA 1.0 mg./day:

During this period the dogs were clinically well. The results for Na^+, K^+, TSP and urine protein excretion as given in Table 1 compare well with values obtained in this laboratory from normal dogs as shown in Table 2. Dog D was slightly hypoproteinemic.

Salt solution only:

During this period the dogs' weights fell from 0.5 to 1.0 kg. and the hematocrit increased in all but dog D which had a relatively high value

TABLE 1. EXPERIMENTAL DATA ON FOUR ADRENALECTOMIZED DOGS ON VARIOUS REPLACEMENT REGIMENS

Dog	Period	Wgt. kg.	Het %	Serum meq./l. Na	Serum meq./l. K	Serum protein gm./100 ml.	Serum gm./100 ml. A	Serum gm./100 ml. G	24 Hr. urine vol. ml.	Urine specific gravity	Urine protein mg./hr.	Urine mg./hr. A	Urine mg./hr. G	Clearance ml. serum/hr. A	Clearance ml. serum/hr. G
A	DCA 1 mg./day	13.0	52	146	5.1	5.5	2.9	2.6	430	1.025	3.5	0.3	3.2	.011	.122
	Salt I.P.	12.5	62	144	6.1	6.1	3.05	3.05	935	1.014	4.1	0.7	3.4	.022	.112
	Low cortisone	11.3	70	130	6.6	8.3	4.3	4.0	200	1.030	3.0	0.5	2.5	.016	.058
	High cortisone	10.8	58	138	6.1	6.6	4.4	2.2	613	1.020	3.1	0.3	2.8	.006	.128
	DCA 1 mg./day	12.3	50	144	3.9	5.0	3.4	1.6	630	1.022	3.9	0.1	3.8	.004	.235
B	DCA 1 mg./day	14.0	53	147	4.5	5.5	3.4	2.1	270	1.013	2.3	0.5	1.8	.016	.084
	Salt I.P.	13.0	64	142	6.8	6.6	3.5	3.1	1090	1.017	4.3	0.7	3.6	.025	.116
	Low cortisone	12.1	63	133	5.7	8.2	3.9	4.3	633	1.018	2.9	0.5	2.4	.012	.056
	High cortisone	12.8	51	144	5.0	6.3	4.5	1.8	1313	1.012	3.1	1.4	1.7	.031	.095
	DCA 1 mg./day	12.8	60	147	5.4	6.6	4.7	1.9	1050	1.012	4.3	1.8	2.5	.037	.134
C	DCA 1 mg./day	13.0	53	157	4.2	6.1	3.7	2.4	560	1.025	4.1	0.4	3.7	.010	.153
	Salt I.P.	12.2	63	139	6.3	6.4	3.7	2.7	1137	1.020	4.7	0.4	4.3	.011	.161
	Low cortisone	11.1	72	134	6.6	5.6	3.2	2.4	510	1.027	5.5	0.5	5.0	.016	.205
	High cortisone	11.3	57	138	4.8	6.5	4.6	1.9	917	1.019	6.2	0.6	5.6	.012	.304
	DCA 1 mg./day	13.0	52	156	5.2	6.3	3.4	2.9	1015	1.024	7.0	0.6	6.4	.019	.222
D	DCA 1 mg./day	15.0	61	150	5.1	5.0	2.2	2.8	530	1.025	6.9	1.6	5.3	.072	.190
	Salt I.P.	14.1	60	150	6.3	5.6	2.5	3.1	1250	1.014	5.8	0.5	5.3	.022	.170
	Low cortisone	12.7	69	137	7.2	6.4	4.1	2.3	1150	1.015	6.2	0.1	6.1	.002	.212
	High cortisone	13.3	55	138	5.9	4.5	2.8	1.7	790	1.019	6.1	0.5	5.6	.019	.335
	DCA 1 mg./day	14.0	53	158	5.4	4.5	2.9	1.6	280	1.040	3.2	0.7	2.5	.026	.153

Averages of data for several days are used for each period except the low cortisone period. The values given in this period are for day 4.

during the preceding period. Serum sodium values fell slightly in 3 dogs and showed no change in one; the potassium values increased over our normal values in all dogs. The serum protein values increased slightly with hemoconcentration, but there was no significant change in urine protein excretion, in the composition of the urine protein or in the clearance of serum albumin or globulin. Associated with the intraperitoneal fluid administration there was an increase in urine volume in all dogs and a decrease in specific gravity of the urine in three.

Cortisone 2.5 mg./kg./day:

During this period of relative hormone insufficiency the body weight of all four dogs continued to decrease, and the hematocrit continued to rise in three dogs, remaining stable but still elevated in dog B. Sodium levels continued to fall in all dogs, and potassium levels increased in three. Dog B showed a decrease in the average potassium value for this period (5.7

TABLE 2. VALUES FOR NA, K, TOTAL SERUM PROTEIN AND URINE PROTEIN EXCRETION OBTAINED IN NORMAL DOGS

	Average	Range	Number of dogs
Na m.eq./l.	153	144 –160	12
K m.eq./l.	5.0	3.8– 5.8	12
TSP gm./100 ml.	6.0	5.3– 7.5	13
Urine protein mg./hr.	3.6	0.5– 8.0	15

m.eq./l.) as compared with the previous period (6.8 m.eq./l.), but showed a very high value (7.7 m.eq./l.) early in the period. No explanation for the simultaneous decrease in potassium and sodium values is apparent. The serum protein values increased in three dogs and showed a slight fall in dog C. There was no change in the excretion of urinary protein, its composition or the clearance of serum albumin and globulin.

Cortisone 10 mg./kg./day:

With this overdose of cortisone, three of four dogs gained weight, and all showed appreciable decreases in hematocrit values. The sodium levels increased and potassium levels decreased in all dogs. In three dogs the serum protein levels decreased, dog D showing a decrease below the initially low serum protein value. Dog C showed an increase from 5.6 gm./100 ml. during the low cortisone period to 6.5 gm./100 ml. during the high cortisone period. This was due to a large increase in the concentration of serum albumin during this period. Two other dogs showed increases in serum albumin (Fig. 1) and all showed significant decreases in serum

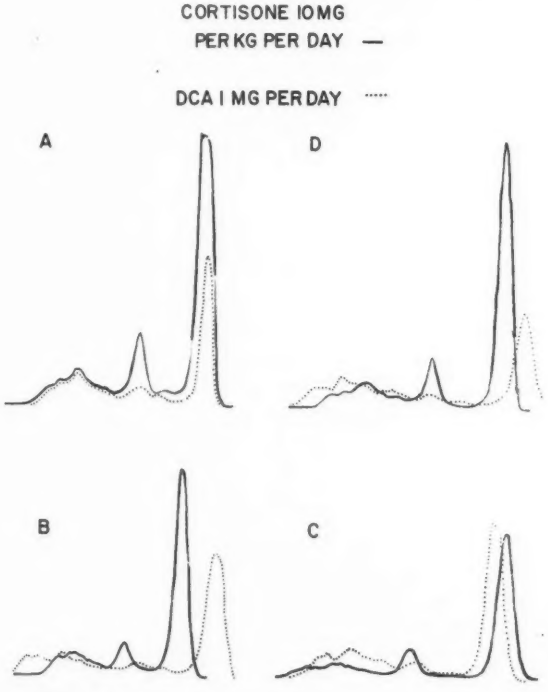

FIG. 1. Electrophoretic patterns of serum protein from four adrenalectomized dogs receiving 1 mg. per day DCA and 10 mg./kg./day of cortisone. Note alpha globulin elevation and relative decrease in globulins in high cortisone period.

globulin. The previously described elevation of serum alpha globulin was noted (Fig. 1), but was relatively small compared to the decrease in the total globulin concentration. The increased serum alpha globulin was not associated with an increase in the urinary excretion of this fraction. Urinary excretion of albumin and globulin was essentially unchanged. The clearance of globulin was elevated in all dogs due to the decreased serum concentration of this fraction.

DCA 1.0 mg./day:

During this period the body weights of all four dogs increased to approximate the values of the previous DCA period. Serum sodium continued to increase in all dogs to reach normal levels. The potassium level decreased in dog A to normal levels and in the other three fluctuated within the normal range. The total serum protein concentrations showed little change. Serum albumin concentrations remained high compared to the initial DCA period in three dogs, and the serum globulin values remained low in the same three dogs. Urine protein excretion values were essentially unchanged in three dogs; in dog D there was a fall from 6.1 to 3.2 mg./hr. which may have been a delayed response to the abnormally low serum protein levels. Clearances of serum albumin and serum globulin were not conspicuously altered.

DISCUSSION

In spite of wide variations in the serum levels of proteins and electrolytes induced by different types and levels of hormone replacement, the adrenalectomized dogs in this series showed only minor alterations in renal excretion of protein. Although there were high concentrations of serum protein associated with hemoconcentration during the salt and low cortisone regimens, there was no significant increase in protein excretion. The previously described elevation of the serum alpha 2 globulins was noted during the high cortisone period. There was no accompanying elevation in the urinary excretion of this fraction as is observed or noted in dogs with sterile abscesses (7). This suggests that alpha globulinuria is dependent on a renal abnormality as well as increased amounts of these small molecules in the serum. There was an increase in the clearance of globulins during the high cortisone period, but this was due to constant urine excretion of the fraction despite a diminishing serum level. It appears clear from these results that the absence of the adrenal cortical hormones does not impair the dog kidney's ability to retain protein nor does a very high dose of cortisone significantly decrease the excretion of protein. These results imply that in the normal dog the adrenal cortical hormones play an insignificant role in controlling the amount of protein excreted by the kidney, and that the proteinuria noted in some "stress" situations is not the effect of adrenal cortical response alone.

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THE SIGNIFICANCE OF THE ANTIDIURETIC HORMONE IN THE RELEASE MECHANISM OF CORTICOTROPIN

D. DE WIED
with the technical assistance of
J. MELCHIOR, TINEKE WIEGMAN AND J. CH. VAN BOON
Department of Pharmacology, University of Groningen, The Netherlands

ABSTRACT

In posterior lobectomized rats the antidiuretic response to hemorrhage, histamine injection and a painful stimulus is markedly depressed. Exposure of such animals to "systemic" stress such as hemorrhage under ether anesthesia, histamine and nicotine injection elicits an increase in plasma free corticosterone levels similar to that found in sham operated controls. The increment in plasma free corticosterone following "neurogenic" stress such as strange environment, sound and pain is significantly reduced. A distinct adrenal hypertrophy is found in posterior lobectomized rats. However, the sensitivity of these adrenals is similar to that of sham operated controls.

Treatment of posterior lobectomized rats with 1 unit of Pitressin tannate for 5 consecutive days decreases water intake to that of intact controls, induces the accumulation of neurosecretory material in the infundibular stem, normalizes adrenal weight and simultaneously restores pituitary response to neurogenic stimuli in posterior lobectomized rats. In dehydrated intact rats, in which the posterior lobe was completely depleted of neurosecretory material the effect of strange environment and of sound on plasma free corticosterone levels is also markedly diminished, whereas the effect of hemorrhage under ether anesthesia and the injection of nicotine equals that of control rats.

It is suggested that the antidiuretic hormone is involved in the corticotropic effect of neurogenic stimuli.

IT IS generally acknowledged that the activation of the hypothalamus is essential for the stimulation of the anterior pituitary gland and the subsequent discharge of its hormones into the general circulation.

Green and Harris (1) postulated that the hypothalamus must secrete an agent or agents which are transported to the adenohypophysis through the hypophyseal portal veins, which carry the blood directly from the hypothalamus to the anterior pituitary.

The nature of the neurohormonal agent responsible for the release of corticotropin (ACTH) from the adenohypophysis is still unknown. A great deal of evidence has been presented that a peptide closely related to those found in the posterior pituitary is the specific corticotropin releasing factor (CRF). Several authors have purified a substance from hypothalamic or neurohypophyseal tissue, which exhibits minor pressor, antidiuretic and

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oxytocic properties, but which is highly capable of stimulating the discharge of ACTH under conditions known to prevent the nonspecific activation of the adenohypophysis (2, 3, 4, 5).

However, exposure of animals to noxious stimuli, such as severe exercise, anesthesia, surgical trauma, hemorrhage, pain, noise, histamine injection and strange environment results in a marked antidiuretic response (6, 7, 8) and an increase in the antidiuretic (9, 10, 11, 12) and adrenocorticotropic activities (13, 14) of the blood.

The close association between the antidiuretic and adrenocorticotropic responses to noxious stimuli led to the postulate that the antidiuretic hormone (ADH) is involved in the release mechanism of ACTH (8, 15). In support of this hypothesis is the rapidity with which ADH is released into the circulation of animals exposed to noxious stimuli (7, 8) and the effectiveness with which exogenous ADH induces the release of ACTH from the adenohypophysis in rats with hypothalamic lesions which prevent the stress-induced discharge of ACTH (16, 17).

This report describes studies on the release of ACTH from the anterior pituitary in rats in which the antidiuretic hormone content of the hypothalamic-neurohypophyseal system was varied by the removal of the posterior lobe, the injection of a long acting vasopressin preparation into posterior lobectomized rats and by dehydration of intact animals.

MATERIALS AND METHODS

In all experiments male rats weighing from 120–180 grams were used. Complete hypophysectomy and posterior lobectomy were performed via the parapharyngeal route. The posterior lobectomized animals were treated with streptomycine during 4 days following the operation to eliminate infection of the adenohypophysis and used between 7 and 10 days after surgery. The pituitary gland was inspected macroscopically for completeness of the posterior lobe removal and for possible damage to the adenohypophysis. Sham operation was performed by exposing the base of the skull without further manipulation.

In order to investigate the ability of the posterior lobectomized rat to release ADH, the discharge of the hormone was measured under different experimental conditions and compared to that of sham operated controls. Antidiuretic activity of plasma was determined in the alcohol anesthetized rat (12). The antidiuretic activity of the plasma was expressed in terms of mU. Pitressin per 100 ml. Rats were anesthetized with 3 mg. sodium pentobarbital per 100 gm. of body weight injected intraperitoneally. As soon as anesthesia became manifest, the animals were injected intravenously with 200 units of Heparine per 100 gm. of body weight. After exposure and cleaning of the right common carotid artery over approximately 1 cm., two ligatures were placed around this blood vessel of which the distal one was tied. By pulling and releasing the proximal ligature, the free flowing blood was obtained via an incision made between the two ligatures. In this way approximately five times 1 cc. of blood was withdrawn in about 90 seconds.

Since the first cc. of carotid blood contained only negligible amounts of ADH, the effect of 5 mg. of histamine phosphate per 100 gm. of body weight on antidiuretic potency of the first ml. of carotid blood was assessed 15 minutes after the intraperitoneal administration of the drug.

In order to study the antidiuretic effect of a neurogenic stimulus, non-anesthetized rats were used. The effect of pain on the urine flow of hydrated rats was studied before and after application of the stress (11). Fasted rats were hydrated by stomach tube with 5 ml. of a 0.2% saline solution per 100 gm. of body weight. This volume was given twice, one hour apart. The animals were placed separately in metabolic cages and the urine excretion was collected following the last gavage every 20 minutes during one hour. The animals were handled and prodded just prior to each measurement of urine volume in order to ensure the emptying of their bladders.

The pain stimulus was produced by a two-minute period of mild electric shocks to the feet through a grid which replaced the floor of the metabolic cage. The stimulus was applied at 20 and 40 minutes following the second gavage.

To study the release of ACTH from the adenohypophysis, "systemic" and "neurogenic" stimuli were employed.

Systemic stress: The intraperitoneal injection of either 1 mg. of histamine phosphate per 100 gm. of body weight or of 0.3 mg. nicotine tartrate per 100 gm. of body weight were used as "stressful" stimuli. The treated animals were decapitated 30 minutes following the injection of these substances. These experiments were performed in rats conditioned to treatment of intraperitoneal injection. Hemorrhage was employed as the stress in a final group of rats by bleeding them to death from the exposed carotid artery while they were anesthetized with ether for approximately 10 minutes.

Neurogenic stress: Animals subjected to handling and environmental change were used. After they had been placed in a cage, they were brought from the rat room to the operating room and subjected for at least 15 minutes to a strange environment (smell of blood and anesthetics, noise of animals and instruments, etc.). They were sacrificed by decapitation. Further, a group of animals was placed in an empty aquarium and were exposed to sound which was produced intermittently by a buzzer during 30 minutes. Then they were decapitated. Another group of animals was subjected to pain by a 2-minute period of mild electric shocks to the feet. This group was decapitated 15 minutes after application of the stress.

As mentioned above, plasma free corticosterone was measured at 15 or 30 minutes following application of the stimulus. This was done because preliminary experiments had shown that the maximal corticoid response following the respective noxious stimuli occurred at these time intervals.

Control rats were obtained by taking only two rats from a cage containing approximately 20 animals. These two rats were killed by decapitation within 30 seconds after removal from the cage. It was assumed that this rapid procedure reduced the pituitary response of handling to a minimum.

To assess adrenocortical activity, blood was collected from the trunk into heparinized glass tubes and plasma free corticosterone content was determined according to the method of Van der Vies *et al.* (18).

RESULTS

The result of the extirpation of the posterior lobe is shown in Figure 1. The pituitary stalk shows the effect of the operative trauma and contains small but supranormal amounts of neurosecretory material, located mainly at the periphery of the cut stalk.

As can be seen in Figure 2, the plasma of the first cc. of carotid blood of intact rats contains approximately 10 mU., whereas the fifth cc. possesses about 9 times more antidiuretic potency per 100 cc. of plasma. Thus, hemorrhage causes a sharp increase in antidiuretic potency of carotid blood

in intact rats. In contrast, posterior lobectomized rats failed to respond to the stimulus of hemorrhage with an increase in the antidiuretic activity of carotid blood plasma.

Subsequently, the effect of an intraperitoneal injection of 5 mg. histamine phosphate on plasma antidiuretic activity of the first cc. of carotid blood was determined in normal and posterior lobectomized rats 15 minutes after the administration of the stimulus (Fig. 3). Whereas the plasma antidiuretic potency increased following histamine injection in intact rats, this increment in activity was absent in posterior lobectomized animals.

FIG. 1 Pituitary stalk of posterior lobectomized rat (7 days postop.). Effect of operative trauma clearly visible. *Hyp* hypothalamus; *men* meningeal tissue at skull base. Bouin, 12μ, chrome-alum hematoxylin (27) without counterstain, 140×.

Figure 4 shows the effect of a painful stimulus on the urine flow of intact and posterior lobectomized rats. In the intact rats urine excretion significantly declined in animals exposed to the stimulus. The antidiuretic response to pain in posterior lobectomized rats was far less than in the intact controls. Only after application of the pain for the second time a small but significant antidiuresis occurred.

Subsequently, the effect of systemic and neurogenic stress was studied on the plasma free corticosterone levels of sham operated and posterior lobectomized rats. Figure 5 depicts the results.

Sham operated and posterior lobectomized rats which were handled very carefully in the rat room served as controls. The plasma free corticosterone level of these two groups were similar. Strange environment caused a considerable increase in plasma corticosterone in the intact animals. In contrast, the same stimulus induced only a moderate but significant rise in the

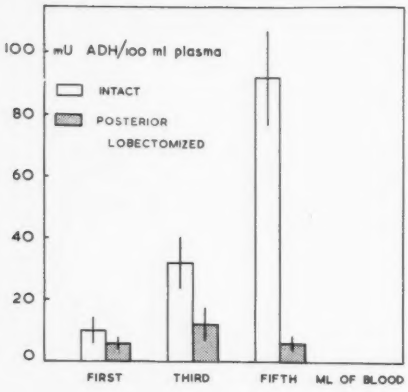

FIG. 2. Effect of haemorrhage on antidiuretic potency of carotid blood. Each bar represents mean of 7 rats (mean ± S.E.) expressed in terms of Pitressin.

plasma corticosterone content of the posterior lobectomized rat. The same was found in rats subjected to sound, and in animals subjected to pain.

In contrast to the foregoing, the effect of systemic stress was not diminished in posterior lobectomized animals. Thus, in a group of rats anesthetized with ether and bled to death from the exposed carotid artery, the plasma free corticosterone content of intact and neurohypophysectomized rats following this procedure rose to 23.7 ± 2.0 and 23.0 ± 3.5 μg per 100 ml. of plasma respectively. The intraperitoneal injection of histamine phosphate or nicotine tartrate also induced similar plasma corticosterone levels in both groups of animals.

Subsequently, posterior lobectomized rats were injected subcutaneously every day with 1 unit of a long acting vasopressin preparation, Pitressin

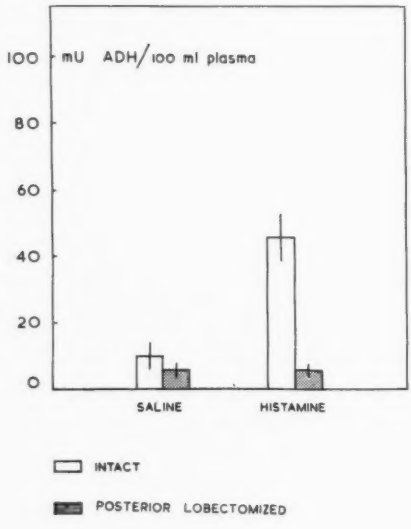

FIG. 3. Effect of intraperitoneal injection of 5 mg. histamine phosphate per 100 grams of body weight and of saline on antidiuretic potency of first ml. of carotid blood. Each bar represents mean of 7 rats (mean ± S.E.) expressed in terms of Pitressin.

tannate.[1] This treatment adjusted the water intake of these rats to pre-operative levels (Fig. 6). Control posterior lobectomized rats received 0.1 cc. of arachis oil during the same time. In this group water intake remained high during the time studied.

Histological examination of the infundibular stem in both groups shows greater amounts of neurosecretory material in rats treated with Pitressin tannate (Figs. 7, 8).

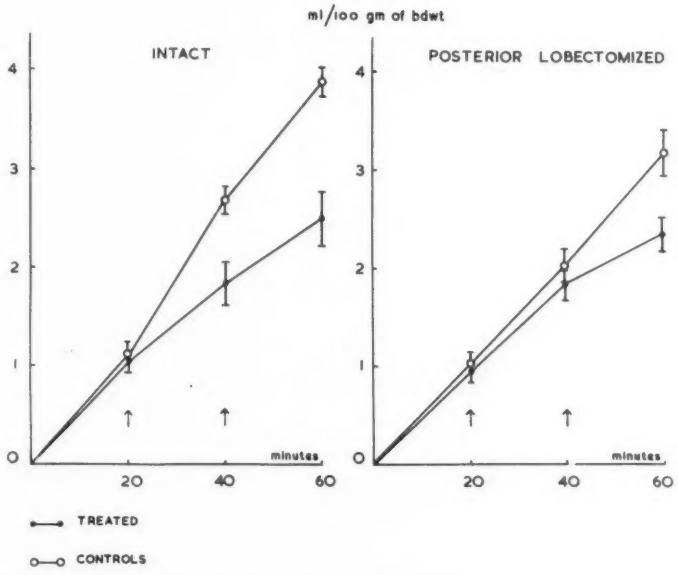

FIG. 4. Effect of posterior lobectomy on antidiuretic response to mild painful stimuli. The curves represent volumes of urine excreted at 20 minute intervals (mean ± S.E.). At times designated by the arrow, one group of rats was exposed to the stimulus (●) while the other (○) was not.

Subsequently, the effect of strange environment and sound on plasma corticosterone was studied in normal and posterior lobectomized rats treated with arachis oil or Pitressin tannate. Neither arachis oil, nor Pitressin tannate interfered with the adrenocorticotropic response to the two stimuli in intact rats (Fig. 9). In posterior lobectomized animals, treated with arachis oil, the effect of noxious stimulation was markedly depressed as could be expected from the foregoing results. However, the treatment with Pitressin tannate for 5 days resulted in a nearly complete restoration of the adrenal response to strange environment and sound.

Nowell (19) and Smelik (20) both reported the occurrence of adrenal hypertrophy in posterior lobectomized rats. Table 1 summarizes the abso-

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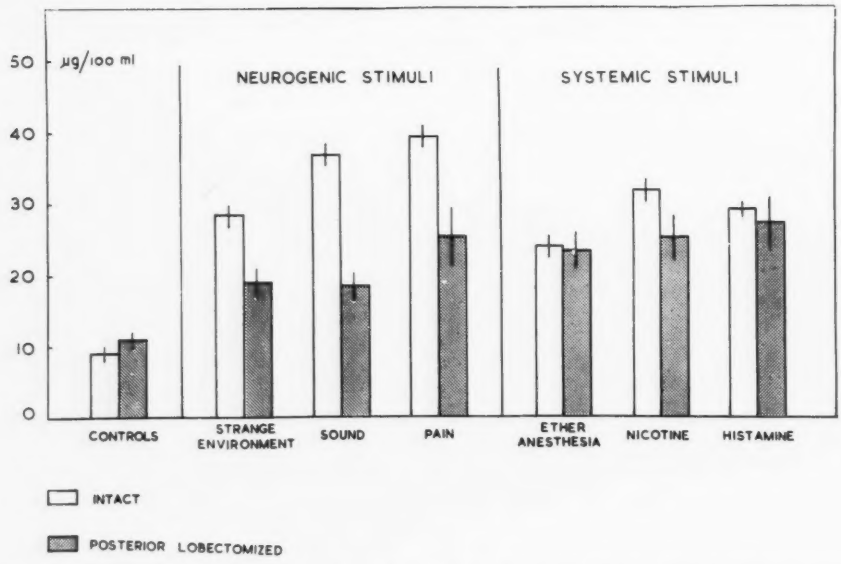

FIG. 5. The effect of neurogenic and systemic stress on plasma free corticosterone of sham operated and posterior lobectomized rats. Each bar represents mean of 12–20 rats (mean ±S.E.).

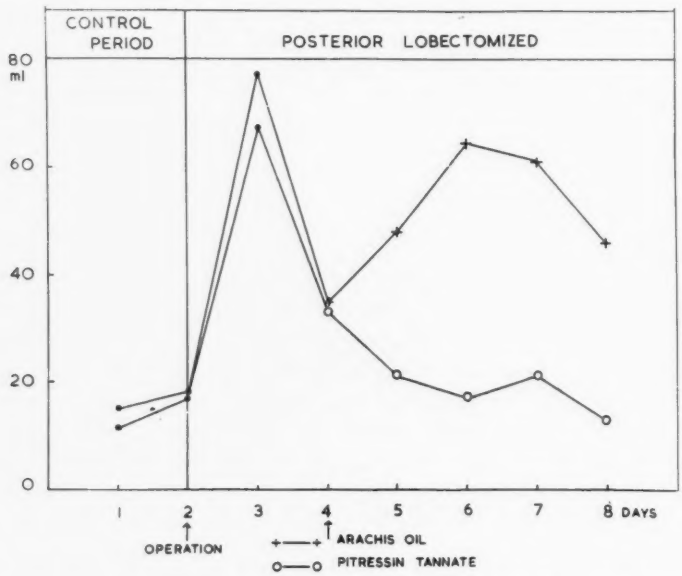

FIG. 6. The effect of arachis oil (0.1 cc. per day—(×)) and of Pitressin tannate (1 unit per day—(○)) on daily water intake of posterior lobectomized rats.

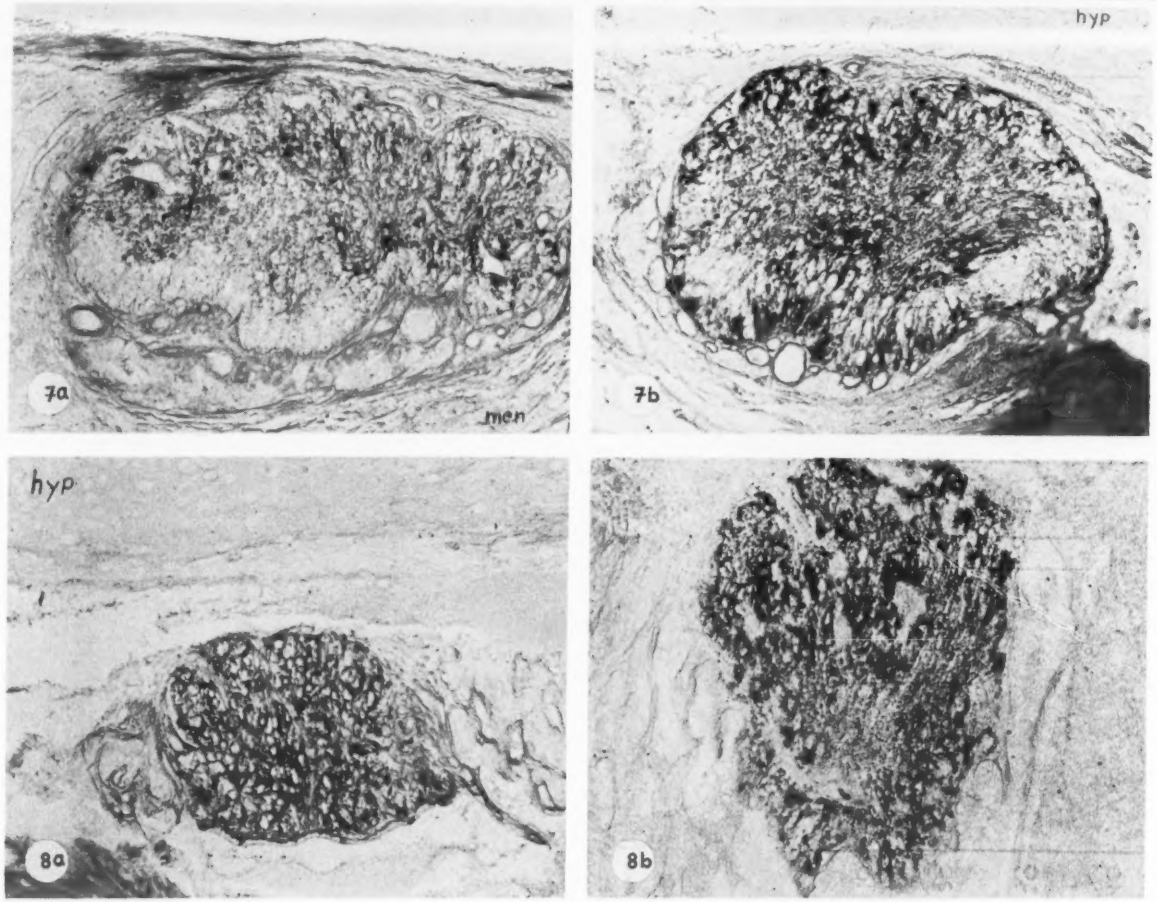

FIG. 7, 8. Pituitary stalk of posterior lobectomized rat (7 days postop.) treated with arachis oil (7a and 8a), or Pitressin tannate (7b and 8b), see text. Abreviations as in Fig. 1. Bouin, 12μ, alcian blue (28) 140×.

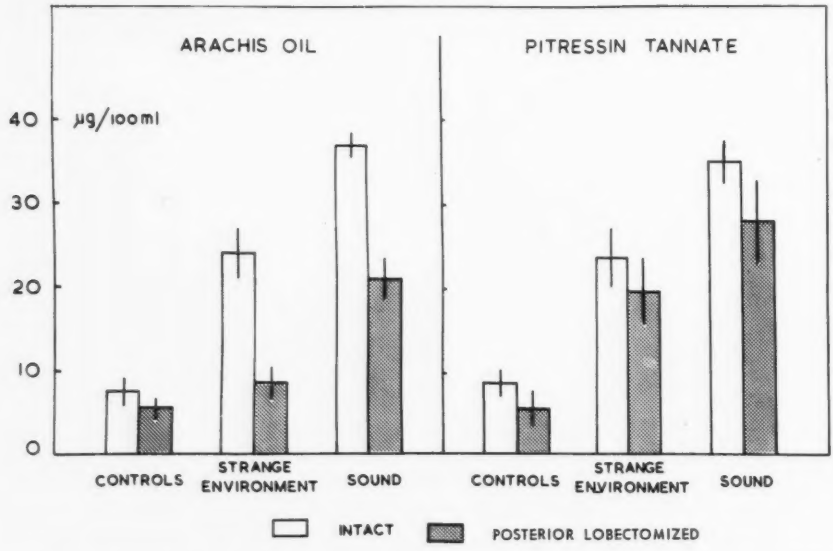

FIG. 9. The effect of neurogenic stress on plasma free corticosterone in sham operated and posterior lobectomized rats treated with arachis oil (0.1 cc. per day) or Pitressin tannate (1 Unit per day) injected subcutaneously for 5 consecutive days. Each bar represents mean of 12–16 rats (mean ±S.E.).

TABLE 1. ADRENAL WEIGHT OF POSTERIOR LOBECTOMIZED AND OF INTACT RATS TREATED WITH ARACHIS OIL OR PITRESSIN TANNATE

	Arachis oil 0.1 ml. per day	Pitressin-tannate 1 unit per day
Intact	30.0 ±1.5* (8)	30.2 ±0.9 (8)
Posterior lobectomized	38.8 ±1.8 (9)	30.8 ±1.6 (9)

* Mean ±standard error of the mean.
Numerals in parentheses indicate number of animals used.

TABLE 2. THE EFFECT OF 2 DOSES OF ACTH ON PLASMA FREE CORTICOSTERONE OF POSTERIOR LOBECTOMIZED AND SHAM-OPERATED RATS TWO HOURS AFTER COMPLETE HYPOPHYSECTOMY

ACTH	Sham operation	Posterior lobectomy
0.2 mu.	6.0 ±0.6* (6)	7.5 ±1.0 (5)
0.8 mu.	13.3 ±1.5 (6)	16.8 ±2.8 (5)

* Mean ±standard error of the mean.
Numerals in parentheses indicate number of animals used.

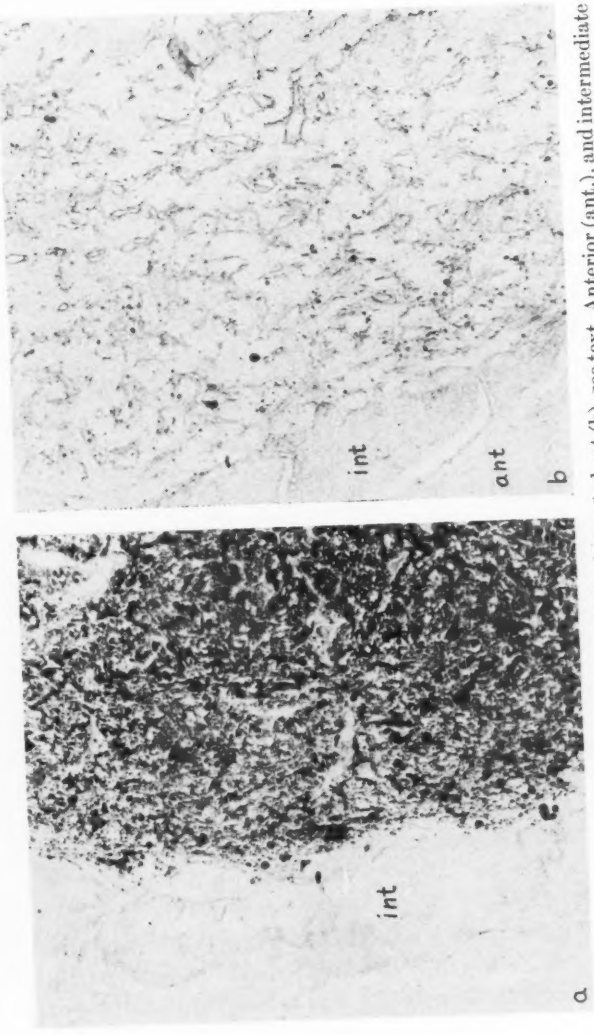

FIG. 10. Posterior lobe of normal rat (a) and of salt treated rat (b), see text. Anterior (ant.), and intermediate lobe (int.) of hypophysis. Bouin, 12μ, aldehydefuchsin (29) without counterstain, 140×.

lute adrenal weights of intact and posterior lobectomized rats. It is clear that the adrenal of the posterior lobectomized animals is significantly greater than those of intact rats. Treatment with Pitressin tannate completely restored adrenal weight towards normal values.

To test the sensitivity of the adrenals of posterior lobectomized rats, the effect of 0.2 and 0.8 mu. of U.S.P. ACTH on plasma free corticosterone levels were studied and compared with that of sham operated controls. ACTH was injected intravenously into one of the lateral tail veins and the

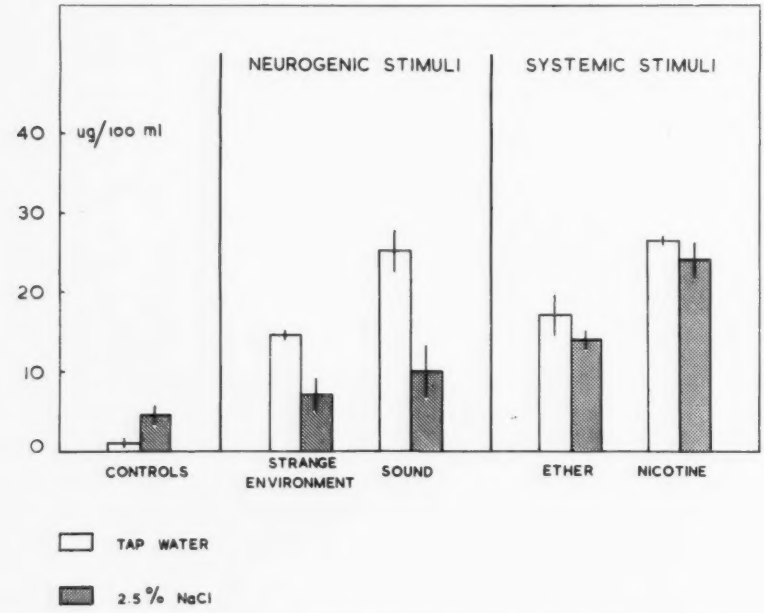

FIG. 11. The effect of neurogenic and systemic stress on plasma free corticosterone of normal and salt treated rats. One group of animals had access to tap water while the other group received a 2.5% saline solution for one week. Each bar represents mean of 10–12 rats (mean ±S.E.).

animals were sacrificed by decapitation 15 minutes after the administration of the hormone. Since the injection itself would cause the endogenous release of ACTH, adrenal sensitivity was assessed in the two experimental groups 2 hours after complete hypophysectomy. As seen in Table 2, the two doses of ACTH elicited nearly identical corticoid responses in the two groups of animals.

Finally, intact rats received a 2.5% saline solution for one week in place of tap water. At the end of this period the posterior lobe had almost completely lost its neurosecretory material (Fig. 10). Subsequently, the effect

of neurogenic and systemic stimuli on adrenocorticotropic activity was investigated.

Whereas animals on tap water exhibited the expected rise in plasma free corticosterone following exposure to a strange environment and to sound, the same stimuli failed to evoke a significant increase in adrenocortical steroids in the salt treated rats (Fig. 11). In contrast, the injection of nicotine tartrate and hemorrhage under ether anesthesia elicited similar adrenocorticotropic activity in the two groups. The saline treatment itself caused some elevation of the plasma free corticosterone.

DISCUSSION

In posterior lobectomized rats the antidiuretic response to systemic as well as to neurogenic stress is greatly reduced (Figs. 2, 3 and 4). In these animals a systemic stress induces an increase in plasma free corticosterone levels which equals that of sham operated controls, whereas the effect of neurogenic stimulation is significantly reduced (Fig. 5). These findings are incompatible with those of Nowell (19). This author failed to observe a diminished adrenal ascorbic acid depletion in posterior lobectomized rats following exposure to a ringing bell during 10 minutes. They are partly in accord with the observation of Smelik (20) in this laboratory, who found that the effect of emotional stimuli on adrenal ascorbic acid depletion was completely abolished in posterior lobectomized rats, whereas the effect of unilateral adrenalectomy was similar to that of intact controls.

The controversy between the results of Smelik (20) who reported the absence of adrenal ascorbic acid depletion in posterior lobectomized rats following sound and our data whereby the same stimulus in the same laboratory induced a diminished but significant elevation of the plasma free corticosterone content, is highly intriguing. Whether this discrepancy can be explained on the basis of a difference in the sensitivity of the two parameters used for the determination of adrenocortical function or to other factors has not yet been elucidated. Smelik (20) originally interpreted his results in support of the hypothesis postulated by Miahle-Voloss (21), that emotional stimuli induce the discharge of ACTH from the posterior lobe, whereas a systemic stimulus causes the release of ACTH from the anterior lobe of the hypophysis. However, the present experiments show a nearly complete restoration of the release of ACTH in posterior lobectomized rats maintained on pitressin tannate. Other experiments (unpublished data) suggested that the amount of corticotropin in the posterior lobe is extremely small; using an *in vivo* assay of corticotropin on the plasma free corticosterone levels of hypophysectomized rats after intravenous injection of the assay material, the activity appeared to be 1.71 milliunit per mg. posterior lobe (95% Fiducial limits 1.48–1.97). These facts argue against the hypothesis that posterior lobe ACTH is solely concerned with the activation of the adrenal gland following neurogenic stimu-

lation. Our data do not agree with the results of Rochefort *et al.* (22). These authors found 20–40 milliunits corticotropin per mg. of posterior lobe tissue. The different characteristics of the incubation method of Saffran and Schally used by Rochefort *et al.* (22) and the plasma free corticosterone method may well have caused this discrepancy.

The division of stresses into neurogenic and systemic varieties may be questionable, since all stresses employed here have a neurogenic component. One could argue that the different corticotropic effect of the two types of stimuli is merely a matter of quantitative difference in intensity of the stress. However, the neurogenic stimuli invariably caused a greater rise in plasma corticosterone than the systemic stresses used in the present experiments. That this different corticotropic effect is the result of a time difference between measurement of the plasma corticoid level following neurogenic and systemic stress is not likely since the effect of strange environment and sound was determined at 15 and 30 minutes respectively and the effect of ether stress and nicotine injection at the same times after application of these stimuli.

Alternatively our results may be explained by assuming a different sensitivity of the adrenals of posterior lobectomized rats to ACTH. Nowell (19) and Smelik (20) have shown that the adrenal glands of such rats are significantly greater than those of intact rats. Same was found in the present experiments. However, it appeared that ACTH elicited identical adrenocortical responses in posterior lobectomized and sham-operated rats. Accordingly, our findings cannot be explained on the basis of a difference in adrenal activity in response to ACTH. This is in accordance with the similar corticoid activity of sham operated and posterior lobectomized rats following systemic stimulation.

As shown, the adrenal weight of posterior lobectomized rats treated with Pitressin tannate was equal to that of intact rats. Apparently, the maintenance of a normal water metabolism prevents adrenal hypertrophy which accompanies posterior lobectomy (19, 20). Whether the diabetes insipidus acts merely as a stressful condition is not known. In this respect it is pertinent to note that in rats rendered diabetic insipidus by hypothalamic lesions in the median eminence and rostrally adjacent regions, adrenal hypertrophy is found simultaneously with an impaired release of ACTH (16).

As shown by the last experiment, animals on saline drinking water had an elevated "basal" concentration of plasma free corticosterone. This could have had also a significant influence on the adrenal response to stress as was assumed for the posterior lobectomized rats. However, for the same reason as stated above, *i.e.*, an identical increase in plasma free corticosterone in dehydrated and intact rats following systemic stress, does not indicate that a depressed adrenal sensitivity to ACTH in animals on saline had caused an impaired corticoid response to neurogenic stimulation.

The above mentioned results seem to indicate that the pituitary response to neurogenic stimuli requires the presence of neurosecretory substance in the hypothalamic-neurohypophyseal system, whereas the response to systemic stimuli seems to be independent of this material.

A maintenance dose of Pitressin tannate in the posterior lobectomized rat causes accumulation of neurosecretory material in the pituitary stalk and simultaneously restores the release of ACTH following neurogenic stimuli. In addition, the absence of neurosecretory material in the hypothalamic-neurohypophyseal system as induced by the chronic administration of a hyperosmotic solution depresses the discharge of ACTH following sound and strange environment. Since the neurosecretory material is closely related to antidiuretic hormone (24, 25, 26) it suggests that the presence of adequate amounts of ADH in the hypothalamic-neurohypophyseal system is obligatory for the release of ACTH following neurogenic stress. Although it was found that posterior lobectomy lowered the secretion rate of ADH, the effect of the treatment with Pitressin tannate on ADH content and secretion was not investigated. However, the greater amount of neurosecretory material in the stalk region of the pituitary after the administration of Pitressin tannate and the absence of neurosecretion following dehydration suggest that the two procedures had varied the ADH content and secretion rate of the hypothalamic neurohypophyseal system. McCann and Haberland (23) have shown that crude extracts from the stalk median eminence region of beef and rat brain produce in rats with hypothalamic lesions a significant release of ACTH. It appeared that vasopressin could account for only 10–20% of the ACTH-releasing activity of stalk-median eminence area. It further was shown that ADH was the main corticotropin releaser present in posterior lobe extracts. Apparently a differential localization of CRF and ADH was found in these two portions of the neurohypophyseal system. If this localization is correct, it provides evidence for the thesis professed that the deficient corticoid response to neurogenic stimuli of posterior lobectomized rats reflects a deficit in ADH release.

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OVARIAN HORMONES AND UTERINE GROWTH: EFFECTS OF ESTRADIOL, PROGESTERONE AND RELAXIN ON CELL GROWTH AND CELL DIVISION IN THE RAT UTERUS[1,2]

SAM BRODY AND NILS WIQVIST

Department of Obstetrics and Gynecology, Karolinska Sjukhuset, Stockholm and Gustaf Vth Research Institute, Stockholm, Sweden

ABSTRACT

The influence of ovarian hormones on the uterine growth of castrated rats was studied. The effects of estradiol-17β, alone or in various combinations with progesterone and relaxin, were investigated by measuring dry weight, total amounts of pentose and deoxypentose nucleic acids per uterus and deoxyribonuclease activity. These analyses permit a distinction between hypertrophic (increase in cell mass) and hyperplastic (increase in cell number) growth.

A time-study after a single injection of estradiol showed a rather fast response as regards increase in dry weight, nucleic acid net synthesis and increase in the cellular level of deoxyribonuclease activity. These effects, however, with the exception of deoxyribonuclease activity, were of very short duration.

Estradiol under these conditions induced uterine growth both by hypertrophy and by hyperplasia. Simultaneous administration of progesterone had a restrictive effect on both types of growth. Progesterone given prior to estradiol had a pronounced stimulative effect on the hyperplastic activity. Progesterone injected after estradiol exercised uterotrophic effects in terms of both cellular growth and multiplication.

Relaxin in a single dose together with estradiol augmented the hypertrophic capacity of estradiol. Repeated injections of this hormone in combination with estradiol and progesterone caused both hypertrophic and hyperplastic growth.

Estradiol, alone or in combination with the other ovarian hormones, caused an invariable and sometimes considerable increase in the cellular level of deoxyribonuclease activity.

THE uterotrophic effects of the ovarian hormones have been mostly studied by gross morphological methods. These investigations have demonstrated complicated interactions between estrogens and gestagens. According to doses administered and the species under study antagonistic as well as synergistic effects have been recorded (1, 2).

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[2] The following abbreviations will be repeatedly used in this paper: PNA = pentose nucleic acid; PNAP = PNA-phosphorus: DNA = deoxypentose nucleic acid; DNAP = DNA-phosphorus; DNase = deoxyribonuclease.

The growth-promoting effects of the ovarian hormones in terms of hypertrophic (increase in cell size) and hyperplastic (increase in cell number) activity have been dealt with earlier. Thus it could be demonstrated that during pregnancy a 5-fold increase in the size of the human uterus was caused by cell multiplication, and a concomitant 6-fold enlargement was accounted for by increase in cell size (3). Subsequent studies on the rabbit uterus were carried out to evaluate the respective rôles of estradiol and progesterone as regards these different types of growth (4, 5). These analyses indicated that estradiol induces hypertrophic growth exclusively in this animal, whereas treatment with progesterone prior to the administration of estradiol causes hypertrophic as well as hyperplastic growth.

The present investigation was undertaken to ascertain whether these effects of estradiol and progesterone on the rabbit uterus could also be elicited in the rat uterus, or if differences due to species specificity could also be discerned.

In addition to studies on the uterotrophic effects of estradiol and progesterone, we have also examined those of relaxin. The growth-promoting activity of this hormone in terms of uterine increase in weight has been clearly demonstrated (6). The type of cellular growth induced by this hormone is, however, not known. We have here suitable conditions to attack this problem as, in contrast to the conditions obtaining in the rabbit, the ovaries are probably the exclusive source of this hormone in the rat (7, 8).

MATERIALS AND METHODS

Animals. Virgin, female rats of an inbred strain, maintained under standardized conditions and weighing 180 to 220 gm., were used in all experiments. The rats were spayed and given 2 to 3 weeks' rest before starting the hormone injections.

The animals were killed by chloroform. The uterine horns were removed, blotted on filter paper, weighed on a Mettler balance and frozen promptly. Generally 4 to 5 uteri were pooled for the determination of the nucleic acids and the DNase activity. Every third uterus was used for evaluating the water content in the respective groups.

Hormone preparations. Estradiol-17β (Schering) was dissolved in propylene glycol and progesterone (Progestin, Pharmacia) in peanut oil. The relaxin preparation[3] used was administered in repository vehicles[3] and assayed 150 guinea pig units/mg. In the first series of experiments (see Table 1) the relaxin preparation was dissolved in 1% L-390 (benzopurpurine-4B) and in the second series (see Table 2) administered in 5% beeswax in sesame oil.

All injections were given subcutaneously at separate sites and in volumes of 0.2 ml. Dosages are indicated in the text.

Analytical procedures. After thawing, the uteri were homogenized in distilled water for 5 minutes with a Bühler homogenizer at 40,000 r.p.m. The tissue concentration was approximately 10%. Precautions were taken to keep the temperature close to 0° C.

PNAP and DNAP were determined as described earlier (9, 10).

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[3] Relaxin preparation W 1164 A, Lot 49, and the repository agents were kindly supplied by Dr. R. L. Kroc, Warner-Chilcott Laboratories, Morris Plains, N. J., U.S.A.

TABLE 1. SUMMARY OF OBSERVATIONS AFTER A SINGLE INJECTION OF ESTRADIOL-17β AND RELAXIN

Group	Treatment[1]	Dry weight per uterus[2]	pg. dry mass per tissue unit[3,4]	μg PNAP per uterus[3]	μg DNAP per uterus[3]	DNase per μg tissue DNAP[3]	DNase per mg. dry weight[3]
1	Untreated castrates	31.3±1.51 (10)	236.4	53.5	85.0	1.36	3.74
2	1 μg estradiol 12 hours before sacrifice	38.1±2.14 (10)	303.8 295.5	76.4 79.9	85.6 79.9	1.21 1.34	2.59 2.95
3	1 μg estradiol 36 hours before sacrifice	42.0±2.00 (14)	233.8 240.7 230.5	117.6 111.2 114.5	118.4 109.6 121.8	2.00 1.84 1.87	5.56 4.97 5.27
4	1 μg estradiol 36 hours before and 0.3 mg. relaxin 12 hours before sacrifice	54.8±5.30 (15)	325.0 329.9 335.1	142.0 145.3 135.2	113.3 110.9 99.7	2.22 2.69 2.29	4.44 5.30 4.94
5	1 μg estradiol 72 hours before sacrifice.	36.8±1.00 (10)	274.3 237.3	63.6 68.7	92.4 94.5	2.08 1.86	4.93 5.00

[1] For further data on mode of administration and vehicles, see under Materials and methods.
[2] Mean ± standard error of the mean. Number of animals in group in parentheses.
[3] Analyses performed on pooled material from four to five rats.
[4] For definition, see text.

DNase activity was measured according to a procedure described elsewhere (11). Calf thymus DNA prepared according to Hammarsten (12) was used as substrate. The reaction was stopped by adding trichloroacetic acid-lanthanum acetate (13). After centrifugation the increase in deoxypentose concentration in the supernatant fluid was determined colorimetrically (10), and from this the amount of degraded DNA, expressed as DNAP, was calculated. The DNase activity is expressed as μg DNAP liberated per hour per μg tissue DNAP or per mg. dry weight.

The water content of the tissues was determined by drying the uterine samples to constant weight at 105° C.

The tissue unit has been used for expressing changes in dry mass on a cellular level. This unit has been defined earlier (3) as the average amount of extracellular and intracellular tissue per average amount of DNAP per nucleus which in a wide variety of

TABLE 2. SUMMARY OF OBSERVATIONS AFTER REPEATED INJECTIONS OF ESTRADIOL-17β, PROGESTERONE AND RELAXIN

Group	Treatment[1]	Dry weight per uterus[2]	pg. dry mass per tissue unit[3,4]	μg PNAP per uterus[3]	μg DNAP per uterus[3]	DNase per μg tissue DNAP[3]	DNase per mg. dry weight[3]
1	Untreated castrates	31.3±1.51 (10)	236.4	53.5	85.0	1.36	3.74
2	Oil day 1, 2, 3; 10 μg estradiol day 4, 5, 6	65.7±4.00 (12)	367.2 345.8	206.0 184.7	122.0 111.3	2.95 3.15	5.22 5.92
3	10 μg estradiol day 1, 2, 3; oil day 4, 5, 6	41.9±1.70 (12)	285.1 254.9	77.1 76.6	98.3 101.2	2.47 2.07	5.63 5.28
4	10 μg estradiol day 1, 2, 3; 5 mg. progesterone day 4, 5, 6	55.8±5.30 (11)	303.8 319.0	120.1 128.5	117.9 123.3	2.49 2.68	5.33 5.71
5	5 mg. progesterone day 1, 2, 3; 10 μg estradiol day 4, 5, 6	76.3±2.40 (10)	306.6 329.9	224.5 253.4	158.1 158.0	3.26 3.08	6.91 6.07
6	10 μg estradiol, 5 mg. progesterone, beeswax day 1, 2, 3, sacrificed day 4	51.7±2.40 (7)	328.3 311.0	161.1 142.6	106.7 101.4	2.65 2.69	5.25 5.62
7	10 μg estradiol, 5 mg. progesterone, 5 mg. relaxin day 1, 2, 3, sacrificed day 4	78.8±5.30 (8)	361.1 391.5	265.3 261.0	130.1 141.6	3.52 3.33	6.33 5.53

[1] For further data on mode of administration and vehicles, see under Materials and methods. When not otherwise stated, the rats were sacrificed on day 7.
[2] Mean ± standard error of the mean. Number of animals in group in parentheses.
[3] Analyses performed on pooled material from four to five rats.
[4] For definition, see text.

material has been found to be 0.65 pg. (1 pg. $= 10^{-12}$ gm.). The calculation of this unit is based on the total dry mass and DNAP content per uterus.

Statistical analysis has been performed according to conventional methods (14).

RESULTS

Effects of a single injection of estradiol and relaxin

A time-study was performed on the effects of 1 μg of estradiol, combined with 0.3 mg. relaxin in L-390. A summary of experimental data is given in Table 1. Untreated castrates serve as controls.

Dry weight per uterus. An increase in this respect was noticeable 12 hours after the administration of estradiol. This increase was more pronounced at 36 hours, whereas a decrease was observed at 72 hours. Relaxin given 24 hours after estradiol and 12 hours prior to sacrifice brought about an additional increase in dry weight, the maximal values of the time series being found in this group of rats.

Dry mass per tissue unit. An increase was observed at 12 hours, whereas the mean values for groups sacrificed at 36 and 72 hours seemed to be in the same range as those noted for castrated controls. The group receiving relaxin in addition to estradiol showed the highest values.

PNAP per uterus. The total amount of this cellular constituent per uterus exhibited an increase over the control level at 12 hours after the injection. An additional increase was observed at 36 hours, whereas a decrease was noticed at 72 hours. The group of rats receiving relaxin showed the highest values also as regards this cellular constituent.

DNAP per uterus. No increase over castrate values was found 12 hours after the estradiol injection. A definite increase was observed at 36 hours and a decrease was noticed at 72 hours. Administration of relaxin seemed to have no definite influence on the net synthesis of DNA.

DNase activity. The pH optimum of rat uterus DNase was close to 5.40. The time-activity graph showed a lag phase, which is in agreement with findings on human placenta (11), rat liver (15, 16) and rabbit uterus (4, 5).

When calculated per mg. dry weight the enzyme activity exhibited a decrease 12 hours after the administration of estradiol. An elevation over castrate values was observed in all the other groups with rather small differences between the groups.

When calculated per μg tissue DNAP no change in DNase activity was observed until after the lapse of 36 hours. The increase was also noted at 72 hours. The highest levels were found in the group receiving relaxin.

Effects of repeated injections of estradiol, progesterone and relaxin

The effects of large doses of these ovarian hormones given in various combinations were studied and the analytical results are presented in Table 2.

Dry weight per uterus. Ten μg of estradiol daily for three days caused a

somewhat more than 100% increase in the dry weight of the uteri as compared with castrate values. This effect faded rather quickly, however, and if the animal was killed four days after the last estradiol injection the dry weight was only slightly elevated over that found in castrates (compare Groups 1 and 3, Table 2). If progesterone in a dose of 5 mg. daily for three days was administered after pre-treatment with estradiol according to the above scheme, considerably increased dry weight values were observed as compared wlth those found in castrates (compare Groups 1 and 4, Table 2). Administration of progesterone prior to estradiol accentuated this tendency considerably (Group 5, Table 2). When progesterone was administered simultaneously with estradiol, a restrictive effect of the former hormone on the estradiol-induced increase in uterine dry weight was observed (Group 6, Table 2). The injection of relaxin in 5% beeswax in a dose of 5 mg. daily for three days together with estradiol and progesterone caused the highest dry weight values observed in this study (Group 7, Table 2).

Dry mass per tissue unit. The highest value was observed in the group of rats receiving a combination of estradiol, progesterone and relaxin. Almost as high values were found if the rats received estradiol alone and were killed the day after the last injection. In the group where estradiol-administration was discontinued for four days before sacrifice the dry mass per tissue unit was only slightly elevated over castrate values. Progesterone following estradiol treatment acted synergistically and caused an increase in the dry mass per tissue unit (compare Groups 3 and 4, Table 2), whereas progesterone prior to estradiol had a restrictive effect (compare Groups 2 and 5, Table 2).

PNAP per uterus. This cellular constituent was elevated 4-fold by estradiol in the doses employed here. The effect had disappeared almost completely four days after stopping estradiol administration. Progesterone following estradiol sustained the effect of the latter hormone to a certain extent. Progesterone administered prior to estradiol seemed to have a potentiating effect. When progesterone was administered simultaneously with estradiol, a suppressive effect was noticed. The highest value, a 5-fold increase, was observed in the group receiving a combination of the three ovarian hormones.

DNAP per uterus. An increase over castrate levels was observed in all groups. An intermediate effect was noticed in the group receiving estradiol alone and killed the day after the last injection. When the animals were sacrificed four days after estradiol-administration a decline was found. Progesterone given after estradiol had a stimulative effect. This effect was even more pronounced when progesterone was administered prior to estradiol. This group exhibited the highest amount of DNAP per uterus.

DNase activity. The enzymatic activity showed almost the same pattern when referred to the tissue DNAP or dry weight. Peak values were found

in the group receiving a combination of the three ovarian hormones. In the group receiving progesterone prior to estradiol the values observed were in the same range. In the other groups somewhat lower activity levels were observed, but they were all well above that found in the untreated castrates.

DISCUSSION

The present investigation clearly indicates that estradiol induces a moderate increase in the amount of dry mass per tissue unit. This result indicates that cellular hypertrophy plays a certain rôle in the estradiol-induced growth process. It is also evident, however, that administration of estradiol causes net synthesis of DNA and, as compared with the increase in the dry mass per tissue unit, a percentually equal increase in the total amount of DNAP per uterus takes place. With reference to the above-mentioned constancy in the average amount of DNAP per nucleus, this finding indicates cellular multiplication. From these biochemical data it may therefore be concluded that the growth-promoting effect of estradiol is exercised by hypertrophy as well as by hyperplasia. This is in contrast to the findings in the rabbit uterus, where hypertrophic growth was exclusively induced by estradiol (4, 5).

The interactions between estradiol and progesterone are largely influenced by the time-relationship of the administration of these hormones. Most earlier studies on the effects of ovarian hormones have been carried out without particular reference to the cyclic nature of ovarian hormone-secretion. The biological importance of these rhythmic events stresses the desirability of investigating not only the influence of absolute and relative doses, but also the effect of varied sequence of administration of the different hormones.

When progesterone is given simultaneously with estradiol a suppressive effect on growth is noticed. This is observed as regards all parameters studied. When progesterone is administered prior to estradiol a pronounced synergistic effect on the net synthesis of the nucleic acids, particularly DNA, is observed. On the other hand a decrease in the dry mass per tissue unit is found in this group of rats. These results, taken together, indicate that under these experimental conditions progesterone augments the hyperplastic effect of estradiol. These observations are in line with previous findings concerning the effect of progesterone on the growth of rabbit uterus when this hormone was administered prior to estradiol (4, 5). With this experimental design considerable hyperplastic growth was induced.

The growth-promoting effect of relaxin on the rat uterus is quite obvious. Its qualitative as well as quantitative effects seem, however, to depend on several factors. If relaxin is administered in a single dose of 0.3 mg. together with estradiol, a stimulation of only the hypertrophic type of growth occurs. An increase in the dry mass per tissue unit is here associated with a constant total amount of DNA per uterus. When repeated large doses of

relaxin are injected together with estradiol and progesterone, effects in terms of both hypertrophic and hyperplastic growth are observed. Administered under these conditions, the relaxin causes an increase in DNA net synthesis as well as in the amount of dry mass per tissue unit.

In this connection it should be mentioned that the hormone-induced changes are over-all effects recorded on whole uteri comprising both the endometrial and myometrial layers. As has been demonstrated earlier with special reference to the nucleic acids (18), these tissues show a discriminate response to the influence of these hormones. It may well be that the interactions among the various ovarian hormones observed are due to selective effects on these two types of tissue, and that the recorded antagonistic and synergistic effects may be explained by a selective inhibition or stimulation of either of these tissues. Additional histological studies and biochemical investigations on separated myometrial and endometrial tissue may clarify this point.

The results on the DNase activity presented here confirm the repeatedly demonstrated finding that under normal conditions of growth DNA net synthesis is invariably associated with an increase in the cellular level of DNase activity (11, 15, 16, 19, 20). This observation has earlier been interpreted as indicating that DNase might be involved in mechanisms controlling and regulating the extent of DNA net synthesis (21, 22). The findings reported here are in line with this interpretation.

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ANDROGEN ON PROSTATE BIOSYNTHETIC REACTIONS[1]

WELLS E. FARNSWORTH AND J. RICHARD BROWN

Medical Research Laboratories, United States Veterans Administration Hospital, and University of Buffalo, School of Medicine, Buffalo, New York

ABSTRACT

The object of this study is to find where and how androgens effect and maintain prostatic growth (protein synthesis) *in vitro*. It is shown that the site of action is not the metabolism of citric acid since this material does not accumulate remarkably within the cell. The finding that the rates of not only citric acid synthesis but also of fatty acid and protein production are greater in glands from intact than castrate animals and the additional discovery that the syntheses of fatty acid and protein are stimulated by 10^{-7} M testosterone have evoked the proposal that the hormone facilitates activation of acyl residues by adenosine triphosphate (ATP), the first step in all three biosynthetic activities. In support of this it was found that the synthesis of fatty acids from pyruvate, which does not require ATP for its activation, is insensitive to 10^{-7} M testosterone.

ELABORATE hypotheses have been developed by earlier workers to relate the hormonal dependence of the prostrate to some block in citrate degradation and/or enhancement of citrate synthesis. Davis, Meyer and McShan (1) and Williams-Ashman (2) have demonstrated the presence of most of the enzymes of the Krebs cycle in rat prostate and shown that the levels of activity of most of them are markedly affected by castration and by androgen and estrogen injections. In addition, Williams-Ashman (3) has shown the synthesis and degradation of citric acid by prostate residues and the presence in these preparations of pyruvate and α-ketoglutarate dehydrogenase activity. If citrate degradation is really lacking in an organ endowed with all the aforementioned enzymatic machinery, one must postulate the action of a metabolic inhibitor which is removed in the preparation of the cell-free systems. The following studies performed in our laboratories, some of which were reported recently (4), indicate that:

(a) there is probably no interference with citrate degradation,
(b) there is no intracellular accumulation of citric acid,
(c) the most likely site of action of androgen is acyl activation *per se*, or some process upon which it depends.

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MATERIAL AND METHODS

Minces of pooled ventral prostate tissue from 300–400 gm. male rats were used for all of these experiments. After killing the animals by a blow on the head, the prostates were rapidly excised and collected in ice cold Ringer's solution. The ventral prostates were dissected free of other tissue and from their capsules in iced Ringer's solution under a binocular microscope. The cleaned glands were pooled in a minimum volume of fluid and minced thoroughly with scissors. Suspension of the mince in 50 ml. of Ringer's solution one to three times and centrifugation at 2500 rpm in a refrigerated centrifuge washed the tissue free of extracellular citric acid, broken cells and other debris.

The addition of testosterone to reaction mixtures was done as follows: An appropriate portion of a stock solution of the steroid in ethanol was diluted with ether so that the desired amount of steroid was contained in 1 ml. of ether-alcohol solution. The solution was pipetted into the dry reaction vessels and permitted to evaporate to dryness before the tissue and aqueous reactants were added. An ether-alcohol solution containing no steroid was added to the control vessels in the same way. Tests with radio testosterone indicated that the steroid was completely taken up in the aqueous reaction mixtures used.

Citric acid was determined on a trichloroacetic acid (TCA) filtrate by a modification of the method of Dickman and Cloutier (5) as follows: Except where studies of intra- vs. extra-cellular levels of citric were under consideration, the isolated minces were homogenized in their suspending medium and deproteinized by adding 1 volume of 100% TCA to 9 volumes of homogenate. After centrifugation, the supernate was neutralized with sodium hydroxide, supplemented with about 100 mg. sodium carbonate, and treated with a solution of $BaCl_2$ to precipitate the citric acid. After centrifugation, the precipitate was dissolved in HCl. The barium was removed by treatment of the solution with H_2SO_4 and centrifugation. After washing the residue, aliquots of the supernatant plus washings were taken for citric acid analysis.

Fatty acid synthesis was measured as the rate of incorporation of radioactivity from acetate-2-C^{14} into long chain fatty acids, essentially as described by Nyden and Williams-Ashman (6). The fatty acids were dissolved in a scintillation "cocktail" consisting of 0.4% PPO (2,5-diphenyloxazole) and 0.005% POPOP (p-bis-(2-(5-phenyloxazoyl))-benzene) in toluene and counted in a Packard Liquid Scintillation Spectrometer.

Protein synthesis was assayed in terms of the rate of incorporation of radioactivity from S^{35}-cystine, C^{14}-glycine or C^{14}-dl-leucine. Following incubation, the protein was repeatedly precipitated with TCA and redissolved in 0.4 M KOH containing 10 μmole

TABLE 1. EFFECT OF FLUOROACETATE ON CITRICOLYSIS IN THE VENTRAL PROSTATE

Trial No.	Citric con., μgm./mg.		Diff.
	Control	Treated	
1	106.3	205.0	+ 98.7
2	97.0	166.1	+ 69.1
3	115.9	139.7	+ 23.8
4	153.0	272.5	+119.5
5	128.2	200.0	+ 71.8
Mean	120.1	196.7	+ 76.6
			t = 3.95
			P = 0.02

Minces incubated in Ringer phosphate buffer containing 0.001 M K acetate, 0.001 M K oxaloacetate. Fluoroacetate added = 0.001 M. Incubation 2 hrs. at 37° C in 100% O_2.

$$t = \frac{\text{mean diff.}}{\sqrt{[\Sigma D^2 - (\Sigma D)^2/N]/(N-1)}}$$

stable amino acid per tube as a carrier. The final precipitate was washed successively with alcohol and ether and dried in a desiccator. A weighed amount (less than 2 mg.) of dried protein was dissolved in 1 ml. of Hyamine. The solution was diluted with 10 ml. of 0.6% PPO in toluene and counted in the Liquid Scintillation Spectrometer. The TCA filtrate was treated with alkaline dinitrofluorobenzene (DNFB) as described by Porter (7). The excess DNFB was removed from the alkaline reaction mixture with ether. The mixture was acidified and the DNP derivative extracted into ether of which an aliquot was plated for counting with a thin window counter.

Keto acids were isolated as dinitrophenylhydrazones by the method of Cavallini and Frontali (8). The hydrazones were applied to Whatman No. 1 paper and chromatographed in a descending system employing n-butanol saturated with 3% ammonia. The dried chromatogram was scanned with a Nuclear-Chicago Actigraph coupled through a Nuclear-Chicago Rate Meter to an Esterline-Angus recorder.

RESULTS

(1) *Evidence of a fully functional Krebs cycle*

(a) It is known that fluorocitrate, by acting as a competitive inhibitor of citrate in the formation of isocitrate, can cause the blockade of citrate degradation with a consequent accumulation of the citric acid. If, however, some other mechanism is present which also prevents citrate oxidation, one cannot expect the addition of fluorocitrate to produce a greater concentration of the acid. It was found in several experiments that the addition of fluoroacetate to the substrate mixture of oxaloacetate plus acetate produced dramatic increases in the yield of citric acid by prostate minces (Table 1). Even in the absence of fluoroacetate the rate of citric synthesis by prostate minces (about 0.31 μmole/mg. dry weight/hr.) is rapid compared to other tissues. Breusch (9) reported a rate of 0.052 μmole/mg./hr. in kidney minces. Marcus and Elliott (10), with the more concentrated 35–70% saturated ammonium sulfate fraction of pigeon liver powder supplemented with 5 μmoles adenosine triphosphate (ATP), showed a rate of 0.17–0.22 μmole citric acid/mg. protein/hour.

(b) If the Krebs cycle were interrupted at any point, one would not expect to find the incorporation of the radioactivity of C^{14}-acetate into pyruvic or oxaloacetic acid. To test the integrity of the cycle in the prostate, minces were incubated with C^{14}-acetate plus oxaloacetate both alone and together with either fluoroacetate or malonate. After homogenizing the tissue, the supernatant fluid was treated with dinitrophenylhydrazine. The keto acid hydrazones were extracted, chromatographed and scanned for radioactivity. Figure 1 shows a strong pyruvate hydrazone band on the chromatogram of the control group (oxaloacetate and acetate) and that it possesses considerable radioactivity. Chromatograms of the keto acids of the groups blocked at citrate by fluoroacetate or at succinate by malonate lack the pyruvate. Without oxaloacetate, of course, no citrate is formed and hence no pyruvate is produced.

It thus appears that the prostate can metabolize citric acid as well as most tissues. Huggins' report (11) of a low oxidation of citric acid is

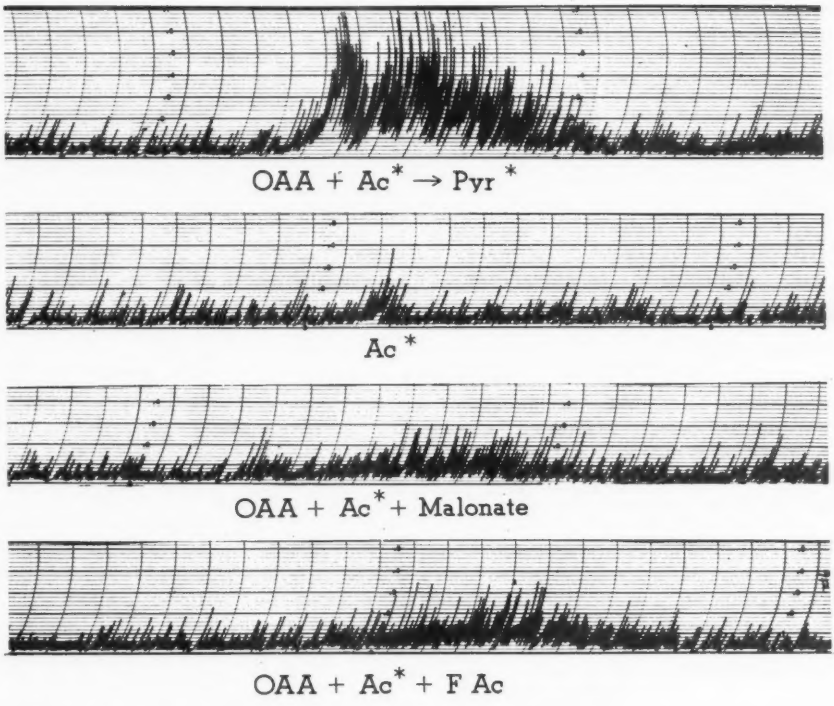

FIG. 1. Pyruvate formation by rat ventral prostate minces. Scan of chromatograms of hydrazones formed from the indicated reaction mixtures. Concentration of reactants: .001 M K oxaloacetate; .001 M K acetate supplemented with 10 μc sodium-1-C^{14}-acetate; .001 M sodium fluoroacetate; .005 M potassium malonate. The active zone shown with oxaloacetate plus acetate has a mobility in the chromatographic system used, butanol saturated with 3% NH_3, identical with authentic pyruvic acid hydrazone.

undoubtedly due to the well known impermeability of intact mitochondria to citric acid.

(2) *Explanation of the high citrate concentration of the prostate*

Analyses of triply washed minces of ventral prostate tissue revealed that the intracellular concentration of citric acid is no more than ten times that reported by Thunberg (12) in blood, liver, kidney, and some of the other organs (Table 2). The washings of the minced tissue are very rich in citrate, indicating that the acid is accumulated extracellularly, presumably in the secretory ducts of the gland.

One can see then that the rate of citric acid synthesis is very high but that essentially all the excess of the acid over that typical of most tissues is removed from the cell as a secretory product. The comparatively stable concentration of citrate within the prostate cell (data of intact and castrate,

TABLE 2. INTRA- AND EXTRACELLULAR CITRIC ACID OF THE VENTRAL PROSTATE

	Extracellular			Intracellular		
	No. analyses	Conc. mg./gm.	S.D.	No. tests	Conc. μgm./gm.	S.D.
Intact	9	1.41	±0.47	9	141	±85
Castrate*	9	0.73	±0.31	2	82	±18

* These animals were sacrificed 3 days following castration.

Table 2) suggests that the secretory mechanism prevents gross concentration or depletion of intracellular citric acid.

(3) *A search for the site of action of testosterone*

(a) Citrate Synthesis. In confirmation of the work of others, Table 2 shows that citrate synthesis is impeded by castration. Similarly, *in vitro* studies show (Fig. 2) that citrate synthesis is dramatically depressed by higher, possibly toxic levels of androgen.

(b) Fatty Acid Synthesis. Upon incubation of minced prostate tissue with levels of testosterone ranging from 10^{-4}–10^{-9} M it was found (Fig. 3) that maximum rates of respiration and incorporation of acetate-1-C^{14} into fatty acids occurred with a 10^{-7} M concentration of testosterone. Confirming earlier work (13), quantities of testosterone in excess of 1 μg (10^{-6} M) were sharply inhibitory. Reduction of the concentration below 10^{-7} M resulted in diminished stimulation of respiration and synthesis.

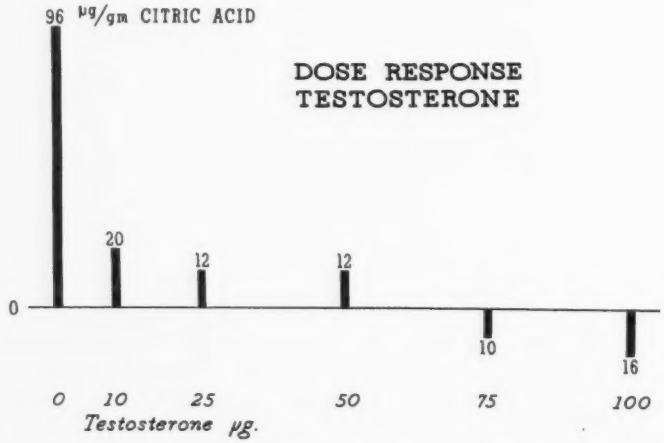

FIG. 2. Testosterone dose level on citric synthesis by washed rat ventral prostate mince incubated 90 minutes with .01 M K oxaloacetate plus .01 M K acetate in Ringer phosphate buffer, pH 7.4. Figures above bars indicate group mean in μg/gm. relative to a control group containing no substrate. Each point is average of 3 or more experiments.

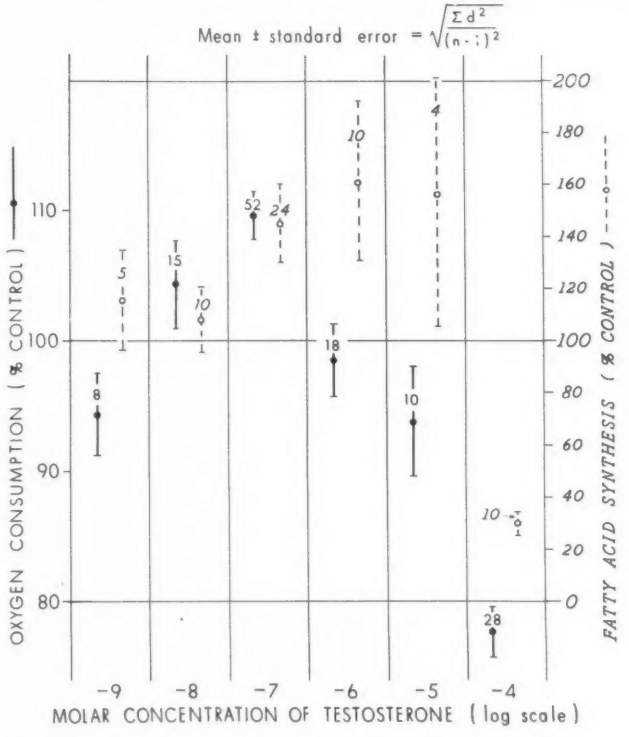

FIG. 3. Testosterone dose level on respiration and fatty acid synthesis by rat ventral prostate mince incubated 90 minutes with .001 M K malate plus .001 M K acetate plus 10 μc sodium-1-C^{14}-acetate in Ringer phosphate buffer, pH 7.4 .

A number of experiments (Table 3) have confirmed the stimulatory effect of 10^{-7} M testosterone on fatty acid synthesis and the lack of any consistent effect on respiration.

(c) Protein Synthesis. Besides acting as a stimulus to the incorporation of acetate into fatty acids, testosterone (10^{-7} M) also accelerates protein synthesis. In a series of experiments (Table 3) both S^{35}-cystine and C^{14}-leucine were incorported into the protein of rat ventral prostate minces. In one experiment, where amino acid levels were also measured, it was found that protein synthesis was at the expense of the amino acid pool (Table 4).

The caudal lobe of one monkey (kindness of Dr. Back of Roswell Park Memorial Institute) was found to respond similarly to 10^{-7} M testosterone (Table 5). There was an acceleration of the incorporation of S^{35}-cystine into the total protein and about an equal increase in acid phosphatase. Caudal lobe tissue from another monkey (kindness of Dr. Ambruss of Roswell

TABLE 3. EFFECT OF TESTOSTERONE (10^{-7} M) ON METABOLISM OF THE RAT PROSTATE

Process	Units	No. expts.	Control	Exptl.	Diff. (Net)	t	P
(1) Respiration	$\frac{\mu\text{moles/mg.}}{90'}$	14	.664	.636	−.028	2	>0.05
(2) Fatty acid	$\frac{\text{cpm/vessel}}{90'}$	24	4153	5481	+1315	3.14	<0.01
(3) Protein	cpm/mg. protein/90′	29	1725	2020	+ 123	2.58	<0.02

Substrate—0.001 M K malate, 0.001 M K acetate in Ringer phosphate buffer pH 7.4. Addition to line 2, 2.5 μc C^{14}-acetate, to line 3, 1 μc radio amino acid (cystine-S^{35}, glycine-C^{14} or dl-leucine-C^{14}).

Park Memorial Institute) incorporated nearly twice as much C^{14}-acetate into long chain fatty acids in the presence of 10^{-7} M testosterone as in its absence. Inability to obtain more tissue from mature monkeys has prevented further confirmation of these findings.

(4) *Acyl activation*

The three processes shown to be stimulated by 10^{-7} M testosterone, viz. biosynthesis of fatty and citric acids and of proteins have a common first step, i.e., activation of the acyl moieties, acetate or amino acids, by the appropriate kinase and ATP.

$$\text{Acid} + \text{ATP} \xrightarrow{\text{kinase}} \text{acyl adenylate} + \text{pyrophosphate.}$$

Lipogenesis and citricogenesis, but not protein synthesis *per se*, require the conversion of acetyl adenylate to acetyl coenzyme A (CoA).

$$\text{acetyl adenylate} \xrightarrow{\text{CoA}} \text{acetyl CoA} + \text{adenosine monophosphate}$$

Acetyl CoA can also be formed, however, from pyruvate without the intervention of ATP.

$$\text{pyruvate} + \text{diphosphopyridine nucleotide (DPN)} + \text{lipoic acid} + \text{thiamine pyrophosphate} + \text{CoA} \xrightarrow[\text{dehydrogenase}]{\text{pyruvic}} \text{acetyl CoA} + \text{reduced DPN (DPNH)}$$

TABLE 4. PROTEIN AND FATTY ACID SYNTHESIS BY VENTRAL PROSTATE MINCES

Constituent	No. expts.	Units	Control	Treated*	Diff. %
1. Amino acids	3	cpm./ml.	177,333	157,167	−11.38
2. Protein	3	cpm./mg.	1,725	2,212	+28.23
3. Fatty acids	4	cpm./ml.	4,020	5,806	+44.42

* Testosterone, 10^{-7} M.
Minces incubated in Ringer phosphate buffer containing, for constituents 1 and 2, 2 μc S^{35}-cystine; for constituents 3, 2×10^{-4} M C^{14}-acetate.

TABLE 5. SENSITIVITY OF MONKEY PROSTATES (CAUDAL LOBES) TO TESTOSTERONE (10^{-7} M)

Monkey no.	Tissue wt.	Test	No. trials	Units	Control	Treated	Diff. %*
1	72.9 ± 6.3	Protein Synthesis	2	cpm./mg. protein	1435	1771	+23.4
		Phosphatase	2	density units/mg.	4.99	6.32	+26.5
2	15.4 ± 2.4	Fatty Acid Synthesis	3	cpm./mg.	64.9	111.6	+72

* Samples are too small to be statistically significant.

Although it was reported earlier (13) that 10^{-4} M testosterone inhibited fatty acid synthesis from pyruvate as well as from acetate, limited data on the influence of 10^{-7} M testosterone on utilization of pyruvate show no significant effect on either respiration or fatty acid synthesis.

DISCUSSION

The foregoing data show the remarkable sensitivity of the prostate to androgen stimulation. They show that, contrary to what earlier investigators have suggested, the site of action is probably not on oxidative processes, e.g., citrate oxidation, but on biosynthetic activities, viz. manufacture of citric acid, fatty acids and proteins. There are at least three points in the metabolic scheme which could limit all three types of synthesis. The most obvious of these is the level of available ATP. But ATP synthesis is dependent upon the functioning of an ATP-generating system, the principal one being the Krebs cycle in the mitochondria. (A less important, energy-wise, source of ATP is glycolysis.) Since the rate of functioning of the Krebs cycle is dependent upon the supply of acetyl CoA, the synthetic machinery can be controlled by any limitation of available CoA or of acetyl-CoA. Finally, of course, a direct action on the enzyme systems concerned with the activation of acyl residues could effect a control on synthesis.

Previous work with a higher androgen level, 10^{-4} M testosterone (13), showed that the androgen depressed respiration and fatty acid synthesis with acetate, adenyl acetate and acetyl CoA. It is not yet known if these activated products respond positively to 10^{-7} M testosterone as acetate and amino acids do.

The failure of pyruvate to respond to 10^{-7} M testosterone may indicate that adenylate formation or utilization is the sensitive site. Further work with pyruvate and studies of ATP and acetyl CoA-turnover rates in progress should help resolve the question of what is the androgen-sensitive site.

The relevance of these *in vitro* studies to an understanding of the metabolism of the prostate *in vivo* is suggested by Grayhack's (14) report of a growth of the gland in response to local, non-systemic androgen treatment

in situ. This report (Grayhack) leads one to believe that the interactions of steroid and tissue observed in the present work are at least qualitatively very much like those which occur in the intact animal.

Whether testosterone is the definitive stimulus cannot be said with certainty since injections of other steroids also have been shown to cause prostate growth. In addition, we have preliminary evidence (15) that testosterone is metabolized by the prostate to such compounds as androstenedione, and various androstane and etiocholane derivatives.

Acknowledgments

We wish to thank Dr. Willard Elliott for the loan of sodium fluoroacetate and instruction in its use, Dr. Wilson D. Langley and Mr. Ted M. Bow for friendly counsel and help, Dr. Irene M. Hulicka for advice on statistical analysis and Mrs. Gail Ann Wilson for expert and interested technical assistance.

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INFLUENCE OF MOLECULAR UNSATURATION ON HORMONAL ACTIVITY PATTERN OF CERTAIN HETEROCYCLIC STEROIDS

ARTHUR L. BEYLER, GORDON O. POTTS AND AARON ARNOLD

Sterling-Winthrop Research Institute, Rensselaer, New York

ABSTRACT

The pattern of hormonal activity in rats of [3,2-c] pyrazole derivatives of steroids was profoundly altered by minor changes in the degree of unsaturation of the steroidal moiety. 17β-Hydroxy-17α-methylandrostano [3,2-c] pyrazole induced marked retention of nitrogen, was myotrophic and weakly androgenic. The singly unsaturated steroid derivative, 17β-hydroxy-17α-methylandrost-4-eno [3,2-c] pyrazole, did not promote nitrogen retention, although it was myotrophic, weakly androgenic and estrogenic. The doubly unsaturated steroid derivative, 17β-hydroxy-17α-methylandrosta-4,6-dieno [3,2-c] pyrazole, was typically estrogenic without anabolic or androgenic activities. The activity patterns of these heterocyclic steroids were compared with those of the corresponding parent steroids: 17α-methylandrostane-17β-ol-3-one, 17α-methylandrost-4-ene-17β-ol-3-one and 17α-methylandrosta-4,6-diene-17β-ol-3-one. The degree of unsaturation of the parent steroids was observed to alter the magnitude but not the kind of hormonal activity. These structure-activity relationships are discussed with reference to known physical chemical features of the various steroids.

INTERESTING and clinically important hormonal activities have been observed for several steroidal [3,2-c] pyrazoles synthesized in our laboratories (1). Among these is 17β-hydroxy-17α-methylandrostano [3,2-c] pyrazole, hereafter referred to as stanozolol,[1] which was observed to be 30 times more active orally than methyltestosterone in improving nitrogen retention of castrated male rats (2). Additional studies (3) indicated that stanozolol administered orally to castrated male rats was twice as myotrophic and $\frac{1}{3}$ to $\frac{1}{4}$ as androgenic as methyletstosterone. The quantitative and qualitative attributes of this new heterocyclic steroid have been confirmed in man by Howard *et al.* (4).

A study of the hormonal activity pattern of several chemical relatives of stanozolol indicated that profound and unexpected modification in the degree and type of activity accompanied the singly and doubly unsaturated homologs; namely, 17β-hydroxy-17α-methylandrost-4-eno [3,2-c]

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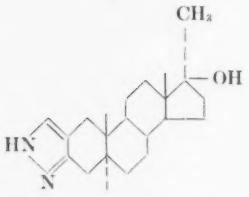
[1] Referred to as androstanazol in previous communications (2, 3).

pyrazole and 17β-hydroxy-17α-methylandrosta-4,6-dieno [3,2-c] pyrazole. This report presents pertinent biological data to point out interesting structure-activity relationships revealed by differences in nitrogen retaining, myotrophic, androgenic and estrogenic properties of these new steroids. Biological data for corresponding parent steroids are included for comparative purposes.

The chemical structures and names assigned to the various steroids included in this study are listed below:

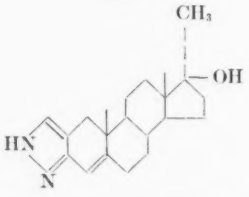
I



17β-Hydroxy-17α-methyl-androstano(3,2-c) pyrazole

Stanozolol

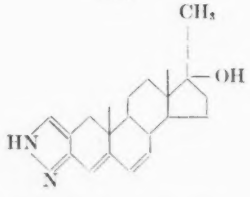
II



17β-Hydroxy-17α-methyl-androsta-4-ene(3,2-c) pyrazole

Hydroxystenazole

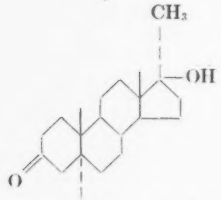
III



17β-Hydroxy-17α-methyl-androsta-4,6-dieno(3,2-c) pyrazole

Methylandrostadienazole

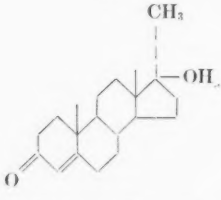
IV



17β-Hydroxy-17α-methyl-androstane-3-one

Methylandrostanolone

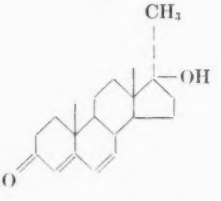
V



17β-Hydroxy-17α-methyl-androst-4-ene-3-one

Methyltestosterone

VI



17β-Hydroxy-17α-methyl-androsta-4,6-diene-3-one

Methylandrostadienolone

PROCEDURES

Assay for Nitrogen Retention. The nitrogen retaining activity of test agents was determined according to the procedure of Kochakian (5) with modifications (2). Castrated male rats of the Sprague-Dawley strain which weighed approximately 200 gm. were placed in individual cages. The initial feed allotment of 14 to 15 gm. per day was decreased gradually until body weights of the rats reached a plateau. Generally, the time required to reach weight and nitrogen equilibrium was 3 to 4 months, at which time the feed allotments were 10 to 11 gm. per day. The percentage composition of the diet was: cerelose, 33.67; dextrin, 33.67; yeast (Fleischman's 2019), 9.2; hydrogenated vegetable oil (Primex), 7.4; lactalbumin, 6.0; methyl cellulose, 5.0; salt mixture (6), 3.7; choline dihydrogen citrate, 0.9; cod liver oil, 0.25; wheat germ oil, 0.16; and liver extract (Wilson's fraction O), 0.05. Bulk was supplied by adding 5 parts alphacel per 100 parts diet. This diet supplied 1.4% nitrogen, equivalent to 9.35% protein. At a feed intake of

10 to 11 gm. per day, the rats received 150 to 175 mg. nitrogen. Control urinary nitrogen values were determined over a 3-day collection period. The urine and daily cage washings were filtered through glass wool into collecting bottles and samples were diluted to 500 ml. for macro-Kjeldahl nitrogen determinations using 25 ml. aliquots. Premedication urinary nitrogen excretion values and body weights provided the basis for the distribution of rats into uniform groups. The test compounds were administered over a 5 day period following the control period. Urine collections were made over the last three days of the medication period to permit two days for onset of response.

Assay for Myotrophic and Androgenic Activities. A modification of the Hershberger, Shipley and Meyer assay (7) was used for determining myotrophic and androgenic activities. The increase in weight of the levator ani muscle of young castrated male rats served as the index to myotrophic activity. Androgenicity was assessed on the basis of increase in weight of the ventral prostate gland. Injections were made daily, except Sunday, beginning one week after castration and continuing for a total of 9 injection days. The weights of the levator ani muscle and the ventral prostate gland were recorded within 24 hours after the last injection.

Assay for Estrogenic Activity. The estrogenic activity of the test compounds was determined according to the cornification response of the vaginal epithelium of castrate rats (8). The material for assay was injected subcutaneously at 2:00 P.M. on day one, and at 10:00 A.M. on day two of the assay period. Vaginal smears were taken with a moistened swab at 4:00 P.M. on day two and at 10:00 A.M. on days three and four. Smears exhibiting a preponderance of cornified epithelial cells were graded as positive. The percent of injected rats in each group which exhibited cornified smears during the assay period was used to quantitate the responses.

Administration of Test Materials. The steroids were administered as solutions or suspensions, depending upon solubility, in cottonseed oil to which ethanol had been added to a volume of 10 percent V/V. In many instances the test steroids were administered orally as well as parenterally. Oral administration was by means of a syringe equipped with a 16 or 18 gauge hypodermic needle with a specially fitted round tip. Parenteral administration was by subcutaneous injection over the scapular area.

Statistical Analyses. The relative potencies with 95% confidence limits were determined according to standard U.S.P. procedures (9).

RESULTS

Nitrogen Retention. Table 1 presents the results of experiments wherein improvement in nitrogen retention of nitrogen equilibrated, castrated male rats was used for assessment of the anabolic property of test steroids. Of the three steroidal pyrazoles tested, only stanozolol significantly increased the amount of nitrogen retained by test animals. This steroid derivative was for all practical purposes as active orally as subcutaneously. It should be emphasized that the only structural difference between stanozolol and the other pyrazole derivatives is the degree of unsaturation of the steroid moiety. Apparently, increased unsaturation of ring A of the steroid nucleus markedly reduced the nitrogen retaining property of steroidal pyrazoles. In contrast, the degree of unsaturation of steroids of the parent compound series, i.e. methylandrostanolone, methyltestosterone and methylandrostadienolone, exerted very little influence on the nitrogen retaining properties regardless of the route of administration.

TABLE 1. THE EFFECT OF VARIOUS STEROIDS ON NITROGEN RETENTION BY CASTRATED MALE RATS

Test compounds	Route of administration	Dose mg./rat/day	No. of rats	Nitrogen retention, increase over control (mg./rat/day)
				Mean ±s.e.
Stanozolol Cpd I	s.c.	0.1	5	1.70 ±1.87
		0.4	7	13.86 ±1.96
		1.6	6	19.42 ±2.24
		6.4	7	25.30 ±3.36
	i.g.	0.1	5	5.38 ±3.11
		0.4	6	10.83 ±4.37
		1.6	7	20.77 ±3.17
		6.4	6	19.50 ±3.10
Hydroxystenazole Cpd II	s.c.	0.632	7	12.2 ±5.7
		1.0	7	0.8 ±4.9
		2.0	7	11.2 ±3.8
		4.0	4	6.1 ±8.5
Methylandrostadienazole Cpd III	s.c.	0.2	6	2.5 ±2.8
		2.0	7	1.5 ±2.6
Methylandrostanolone Cpd IV	s.c.	0.25	6	12.6 ±2.7
		1.00	7	17.8 ±3.6
Methyltestosterone Cpd V	s.c.	0.25	6	10.1 ±5.9
		0.79	6	21.1 ±3.1
	i.g.	6.0	4	5.76 ±2.53
		24.0	7	14.24 ±3.33
		10.0	8	11.2 ±1.7
		40.0	4	20.5 ±1.7
Methylandrostadienolone Cpd VI	s.c.	0.05	5	3.5 ±5.1
		0.158	5	11.7 ±6.0
		0.5	5	15.3 ±4.1
		0.079	5	5.5 ±4.4
		0.25	4	10.4 ±5.6
		0.79	4	17.4 ±3.4
	i.g.	10.0	8	7.9 ±2.8
		40.0	7	14.0 ±3.0

Administered parenterally, all the steroidal compounds studied, except the mono- and bisunsaturated pyrazoles, exhibited comparable degrees of nitrogen retaining activity. A marked difference, however, in degree of oral activity existed among the compounds. Stanozolol was 34(25–45) times more active than methyltestosterone and 94 (limits indeterminate) times more active than methylandrostadienolone. The oral nitrogen retaining activity of methylandrostanolone was not determined.

Myotrophic and Androgenic Activities. The myotrophic and androgenic properties of the six compounds of this study were determined by measuring concurrently the weight responses of the levator ani muscle and the ventral prostate gland of castrated immature male rats. The data from

TABLE 2. THE MYOTROPHIC AND ANDROGENIC RESPONSES OF CASTRATED MALE RATS TO VARIOUS STEROIDS

Compound	Route of administration	Dose mg./kg./day	No. of animals	Myotrophic response, levator ani wgt.	Androgenic response, ventral prostate wgt. (mg.)
				Mean ±s.e.	Mean ±s.e.
None	s.c.	—	52	29.5 ±0.9	5.1 ±0.2
Stanozolol Cpd I	s.c.	1.4	12	35.3 ±1.5	7.3 ±0.4
		2.8	12	43.7 ±2.5	13.0 ±0.7
		5.6	12	47.9 ±2.2	14.5 ±1.0
		11.2	12	72.9 ±4.0	39.6 ±2.8
	i.g.	5.25	6	28.6 ±3.1	4.3 ±0.7
		10.5	6	39.3 ±4.1	8.6 ±0.9
		21.0	18	51.3 ±2.4	13.4 ±0.9
		42.0	12	67.1 ±3.3	24.2 ±2.4
Hydroxystenazole Cpd II	s.c.	2.8	6	32.9 ±1.9	6.5 ±0.8
		5.6	6	36.9 ±3.0	10.7 ±1.9
		11.2	5	50.2 ±3.3	16.2 ±1.8
	i.g.	10.5	6	39.4 ±2.3	6.2 ±0.7
		21.0	6	51.4 ±2.1	9.9 ±0.3
		42.0	6	52.6 ±3.4	14.0 ±1.3
		84.0	6	63.8 ±3.7	16.6 ±1.7
Methylandrostadienazole Cpd III	s.c.	2.8	6	38.6 ±2.7	6.1 ±0.7
		5.6	6	33.1 ±2.0	5.9 ±0.3
		11.2	7	34.1 ±2.6	6.2 ±0.4
		16.8	6	25.0 ±4.5	4.2 ±1.0
Methylandrostanolone Cpd IV	s.c.	0.7	12	40.7 ±1.7	29.5 ±1.7
		1.4	12	46.0 ±2.0	42.9 ±2.8
		2.8	11	50.1 ±2.4	60.4 ±5.3
		5.6	5	60.0 ±6.6	66.4 ±7.9
		11.2	5	74.7 ±5.9	84.3 ±6.6
	i.g.	10.5	6	31.2 ±3.9	17.1 ±1.4
		42.0	6	34.5 ±2.3	39.0 ±1.9
Methyltestosterone Cpd V	s.c.	0.7	12	46.6 ±2.1	31.3 ±2.1
		1.4	12	60.3 ±3.2	43.1 ±3.1
		2.8	6	74.5 ±6.9	59.4 ±4.9
	i.g.	5.25	12	31.6 ±1.7	10.6 ±0.8
		10.5	12	33.0 ±1.5	21.9 ±1.2
		21	12	41.9 ±1.0	32.6 ±1.7
		42	12	48.5 ±2.5	45.3 ±3.2
		84	6	63.8 ±2.2	57.7 ±3.9
Methylandrostadienolone Cpd VI	s.c.	8.4	8	41.4 ±2.6	13.4 ±1.5
		16.8	8	49.0 ±3.4	16.7 ±1.1
	i.g.	42	6	41.1 ±2.6	14.5 ±1.6
		168	6	50.2 ±4.8	21.6 ±2.9

these experiments are tabulated in Table 2. Within the steroidal pyrazole series, stanozolol was the most active myotrophic agent being 1.8(1.6–2.0) times more active subcutaneously and 3.0(2.4–3.5) times more active orally than the singly unsaturated congener. The doubly unsaturated compound was inert. The changes in degree of androgenicity were observed to

parallel the differences in myotrophic activity of the pyrazole compounds.

Activity differences among the three parent compounds were less marked. Methyltestosterone was the most active myotrophic agent followed in rank by methylandrostanolone then methylandrostadienolone. However, methylandrostanolone was equally as androgenic as methyltestosterone. The doubly unsaturated steroid, methylandrostadienolone, was very weakly androgenic.

These relationships indicate that as the degree of unsaturation of the steroidal pyrazoles is increased there is a corresponding decrease in the myotrophic and androgenic properties, with unsaturation in both the 4:5 and 6:7 carbon positions of the steroid moiety of the pyrazole derivative resulting in complete loss of these activities. In the parent compound series, on the other hand, the presence of a double bond in the 4:5 position enhanced the myotrophic but not the androgenic activity, in that methyltestosterone subcutaneously was 9.4(8.1–10.8) times and orally was 2.2 (1.7–2.8) times more myotrophic than methylandrostanolone. In each case the two compounds were equally androgenic. Unsaturation at carbon positions 4:5 and 6:7 lowered both myotrophic and androgenic activities, but decreased the myotrophic activity to a lesser degree.

It should be noted that the degree of androgenicity of the pyrazole derivatives was much lower than that of the corresponding parent compounds, particularly when compared at equally effective myotrophic dose levels. Of interest also is the fact that stanozolol, administered orally, was the most active myotrophic steroid in either series. This compound was 2.2(1.5 to 3.3) times more active than methyltestosterone and 3.8(2.1–7.1) times more active than methylandrostanolone.

Estrogenic Activity. The results of assays of estrogenic activity based on the response of the vaginal epithelium of castrated female rats are shown in Table 3. The singly and doubly unsaturated steroidal pyrazoles were definitely estrogenic within the dose range of 0.5 to 2.5 mg./kg. This observation was unexpected since earlier assays had shown that stanozolol was not estrogenic and since the corresponding parental steroids were known to be devoid of estrogenicity. Since the degree of estrogenicity of methylandrostadienazole was no greater than that of hydroxystenazole, it may be inferred that unsaturation at position 6:7 of the steroidal pyrazole is relatively unimportnat as a determinant of degree of estrogenicity within the steroidal pyrazole series.

DISCUSSION

The comparisons offered above demonstrate to what extent the magnitude and type of hormonal activity of steroidal pyrazoles of androstane parentage are influenced by the degree of unsaturation of the steroid portion of the molecule. The activity profile of the pyrazole derivative of the fully saturated methylandrostanolone was identical to the parent

TABLE 3. THE ESTROGENIC EFFECT OF VARIOUS STEROIDS ON VAGINAL EPITHELIUM OF OVARIECTOMIZED RATS

Test compound	Dose mg./kg./day s.c.	No. of rats	Percent exhibiting vaginal cornification
Stanozolol	0	10	0
Cpd I	5	10	0
	10	10	0
	20	10	0
Hydroxystenazole	0	20	0
Cpd II	0.1	10	0
	0.5	10	10
	2.0	20	60
	4.0	10	100
Methylandrostadienazole	0	20	0
Cpd III	2.0	10	20
	2.5	10	50
	5.0	10	100
Methylandrostanolone	0	10	0
Cpd IV	5	10	0
	10	10	0
	20	10	0
Methyltestosterone	0	10	0
Cpd V	5	10	0
	10	10	0
	20	10	0
Methylandrostadienolone	0	10	0
Cpd VI	5	10	0
	10	10	0
	20	10	0

steroid; however, the degree of androgenicity was greatly reduced and the anabolic activity attending oral medication was increased several fold based on relative myotrophic activity. In contrast, the pattern of activity of the doubly unsaturated steroid was completely altered by pyrazole substitution from a compound which was anabolic and androgenic (methylandrostadienolone) to one which was solely estrogenic (methylandrostadienazole). The pyrazole derivative of the mono-unsaturated steroid, on the other hand, exhibited a most provocative combination of hormonal activities. This steroidal pyrazole, designated hydroxystenazole, was estrogenic, myotrophic, and weakly androgenic, but was devoid of nitrogen retaining activity in the rat. Preliminary observations (unpublished) indicate that hydroxystenazole may decrease the efficiency of utilization of dietary protein since a greater proportion of nitrogen available in the diet appeared in the feces of castrated male rats during the period of medication with hydroxystenazole than was found excreted during the control period. Fecal nitrogen was not affected by testosterone treatment under identical conditions. This effect of hydroxystenazole may be characteristic for estrogenic substances in rats in that Leatham (10) observed that diethylstilbestrol under somewhat different conditions increased fecal nitrogen excretion. Also, estradiol was observed in our laboratory to bring about

a moderate but uniform rise in fecal nitrogen values of castrated male rats. The catabolic effect of estrogens in rats is a well documented observation (11, 12, 13) and may be related to the ability of estrogen to divert a portion of the available nitrogenous foodstuffs from metabolic pathways to alimentary elimination. It should be noted, however, that estrogens have been reported to be anabolic in certain other mammals such as cattle (14, 15) and humans (16). In connection with this point hydroxystenazole was observed in our laboratory to increase substantially the amount of nitrogen retained by female rhesus monkeys maintained on constant daily caloric and nitrogen intake. Clinical observations by Furman *et al.* (17) indicate that hydroxystenazole is anabolic in male and female human subjects at oral dose levels of 1 to 16 mg./day.

Within the series of steroidal pyrazoles, the degree and type of hormonal activity was profoundly influenced by unsaturation in ring A and B of the steroidal portion of the molecule. Conjectures as to the factors contributing to these changes may be of more than passing interest. Increasing the degree of unsaturation in rings A and B increases the total electron density of the pyrazole ring, increases the lability (electrophilic property) of the hydrogen atom bonded to pyrazole nitrogen and flattens the molecule about the centers of unsaturation. These molecular features tend to make the pyrazole and ring A end of the molecule more similar to phenolic estrogens in terms of configuration and chemical reactivity. Thus, the tautomeric hydrogen on the pyrazole ring becomes more "acidic" and more available for chemical interaction analogous to the acidic hydrogen of the phenolic hydroxy group of natural and synthetic estrogens. The fact that hydroxystenazole was anabolic and androgenic as well as estrogenic suggests that the [3,2-c] pyrazole group is a multifunctional substituent which influences the type and degree of hormonal activities of steroids of androstane parentage to an extent which is in some way related to the presence or absence of molecular unsaturation in ring A of the steroid moiety.

Since the doubly unsaturated steroidal pyrazole was only estrogenic, it is suggested that the anabolic and androgenic features of the molecule have been lost due to molecular distortion and flattening imposed by the steroidal diene system. An indication as to the degree of molecular distortion conferred on the steroid by the 4,6-diene system is obtained from a comparison of the optical rotation measurements for the mono- and bisunsaturated steroidal pyrazoles. The $[\alpha]_D^{25}$ values obtained for 1% solutions of each compound in pyridine were $+132.6 \pm 0.2$ and -126.1 ± 0.1 for the mono- and bisunsaturated steroids, respectively. This large shift in optical rotation is typical for the effect of conjugated diene systems of steroids, as shown by Barton and Klyne (18), and is best explained by induced molecular distortion.

Although these attempts to explain structure-activity relationships are highly conjectural, they do help to vizualize possible mechanisms whereby

minor differences in the structure of steroid hormones affect the kind and degree of endocrine activity. Further efforts to substantiate and extend the present observations are expected to be rewarding.

Acknowledgment

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THE NATURE OF HYTAKEROL® (AT-10) AND ITS COMPARISON WITH CRYSTALLINE DIHYDROTACHYSTEROL[1,2]

A. RAYMOND TEREPKA,[3] PHILIP S. CHEN, JR. AND BETTY JORGENSEN

From the Departments of Radiation Biology and Medicine, University of Rochester School of Medicine and Dentistry, Rochester, New York

ABSTRACT

Paper chromatographic analysis of Hytakerol was carried out using reverse-phase systems. Three distinct compounds were isolated, one of which exhibited an ultraviolet absorption spectrum almost identical to crystalline dihydrotachysterol. Mixed paper chromatograms and infra-red spectra, however, revealed that this Hytakerol sterol is *not* dihydrotachysterol.

Five different lots of Hytakerol were spectrophotometrically assayed after chromatographic separation. On the basis of ultraviolet absorbance, four of the five lots contained 2.7–3.0 times *more* of the "dihydrotachysterol-like" sterol than the label indicated. One lot contained considerably less than the others. Evidence is presented suggesting that the Hytakerol sterol is dihydrovitamin D_2 II, an isomer of dihydrotachysterol, with a significantly lower potency.

Historical aspects of "AT-10" preparation and the clinical implications of our findings are discussed.

THE "calcemic" action of calciferol[4] and that of the structurally closely related dihydrotachysterol has been extensively utilized in the treatment of hypoparathyroidism. Although originally suggested as the ideal substitute for parathyroid hormone (1), the commercially available preparation of "dihydrotachysterol" in this country (Hytakerol®) has fallen into disfavor in recent years due to its greater cost and apparently variable potency (2, 3).

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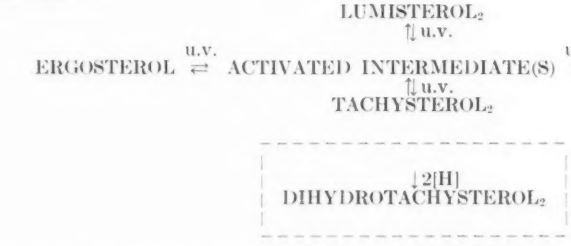
[1] Based on work performed under a contract between the U. S. Atomic Energy Commission and the University of Rochester, administered by the Department of Radiation Biology of the School of Medicine and Dentistry.

[2] A portion of the material presented here appears as an abstract in J. Clin. Invest. **39:** 1035, 1960.

[3] Senior Research Fellow (SF-381), Public Health Service.

[4] The general term "calciferol" refers to any of a number of antirachitic sterols and may be used interchangeably with the term "vitamin D." All are structurally identical except for the chemical nature of the side chain at position 17 in the sterol nucleus. Commonly available forms are: vitamin D_2 = activated ergosterol = ergocalciferol; and vitamin D_3 = activated 7-dehydrocholesterol = cholecalciferol. A subscript 2 is utilized in specifically referring to other related compounds formed during ultraviolet irradiation of ergosterol (e.g., tachysterol$_2$) or a derivative thereof (e.g., dihydrotachysterol$_2$). The subscript 3 pertains in a similar specific way to 7-dehydrocholesterol derived compounds.

Recent studies (4, 5, 6) have suggested that the following sequence of events occuring during the irradiation of a provitamin such as ergosterol is as follows:



It should be noted that whereas vitamin D is a natural irradiation product, dihydrotachysterol, as prepared by von Werder (7), is formed by a separate chemical reduction of tachysterol, another compound which may be isolated from the crude irradiation mixture. When compared with calciferol on an equivalent weight basis both tachysterol and dihydrotachysterol are practically devoid of antirachitic activity (8). The latter compound, however, has a potent calcium mobilizing action (9).

In a comparative study of the relative actions on a milligram for milligram basis of vitamin D, crystalline dihydrotachysterol and Hytakerol in rats, dogs and man (10), it was noted that Hytakerol had significantly less calcium and phosphorus mobilizing activity when compared with pure crystalline dihydrotachysterol. These observations prompted us to investigate the chemical nature of Hytakerol. The present report describes our findings.

METHODS

Paper Chromatography

Four reversed-phase systems of paper chromatography were utilized in this study; the three systems described by Kodicek and Ashby (11), in which the stationary phase is mineral oil, and our own 5% decalin-absolute methanol system. Descending chromatograms on Whatman No. 1 filter paper were run in standard 12×24 inch tanks for 8 to 24 hours depending upon the chromatography system employed. After air drying, the papers were immediately examined under a hand ultraviolet source of 254 mμ to locate the sterols. The sections were marked, cut out, and eluted in a test tube with 3–10 ml. of absolute methanol. A parallel strip from each chromatogram was also stained with antimony trichloride and examined with an ultraviolet lamp of 360 mμ.

Ten to twenty-five μg of a standard solution of crystalline dihydrotachysterol[5] containing 1 mg./ml. was spotted directly onto the papers and used as a reference. On all chromatography systems utilized the crystalline dihydrotachysterol preparation ran as a single spot.

Ultraviolet Analysis

The methanol elutions from the paper chromatograms were read, after appropriate dilution when necessary, in a Beckman DU spectrophotometer. Absorption spectra were

[5] Manufactured by N. V. Philips-Roxane Pharmaceutish-Chemische Industrie "Duphar," Weesp, Holland.

recorded with a Beckman DK-2 recording spectrophotometer. Concentrations of the sterols were calculated from previously determined molar absorbancy values. For dihydrotachysterol $E^{1\%}_{1cm}$ (250.5 mμ) was 1036 which agrees with previously published values (7, 12).

Infra-Red Analysis

Methanol or dichloromethane solutions of the sterols for infra-red analysis were dried in round bottom flasks on a Rinco Rotating Evaporator after adding 50 mg. of potassium bromide which had been screened through a 200 mesh sieve. The residue was scraped from the flask and pressed at 1500 lbs./sq. in. in a Carver Laboratory Press without air evacuation. The pellets were read directly in a Baird-Atomic Model KM-1 infra-red spectrophotometer using a micro-beam holder.

RESULTS

Chromatographic Analysis of Hytakerol

Ten μg of Hytakerol were streaked on one-half of a 7 in. sheet of Whatman No. 1 filter paper. On all chromatography systems employed, three distinct spots could be identified by ultraviolet absorption and $SbCl_3$ treatment. Table 1 shows the running time and the R_f values of these components for each of the chromatography systems. The best separation was obtained on Kodicek A and C systems.

After elution of these spots in a test tube containing 10 ml. of absolute methanol, the ultraviolet spectrum of the eluate was determined against a paper blank of identical size from the opposite half of the chromatography paper. The spectra obtained from these three areas, in addition to the spectrum of crystalline dihydrotachysterol, crude Hytakerol, and sesame oil U.S.P., are shown in Figure 1.

The spectrum of spot I (spectrum D, Fig. 1) corresponded closely to that of crystalline dihydrotachysterol (spectrum C) when the chromatogram was run long enough to prevent contamination with seasme oil (if trace quantities of sesame oil were present the 243 mμ peak was higher than the 250.5 mμ peak and an additional peak due to sesame oil [spectrum B, Fig. 1] was seen at 285 mμ).

TABLE 1. CHROMATOGRAPHIC R_f VALUES OF ULTRAVIOLET ABSORBING MATERIALS IN HYTAKEROL

	Chromatography system			
	Kodicek			5% Decalin: 100% methanol
	A	B	C	
Time (Hrs.)	22	13	9.5	5
Spot I*	.16	.42	.40	.22
II	.28	.59	.75	.71
III	.82	.74	.80	.81

* This spot exhibits an ultraviolet absorption spectrum almost identical to crystalline dihydrotachysterol.

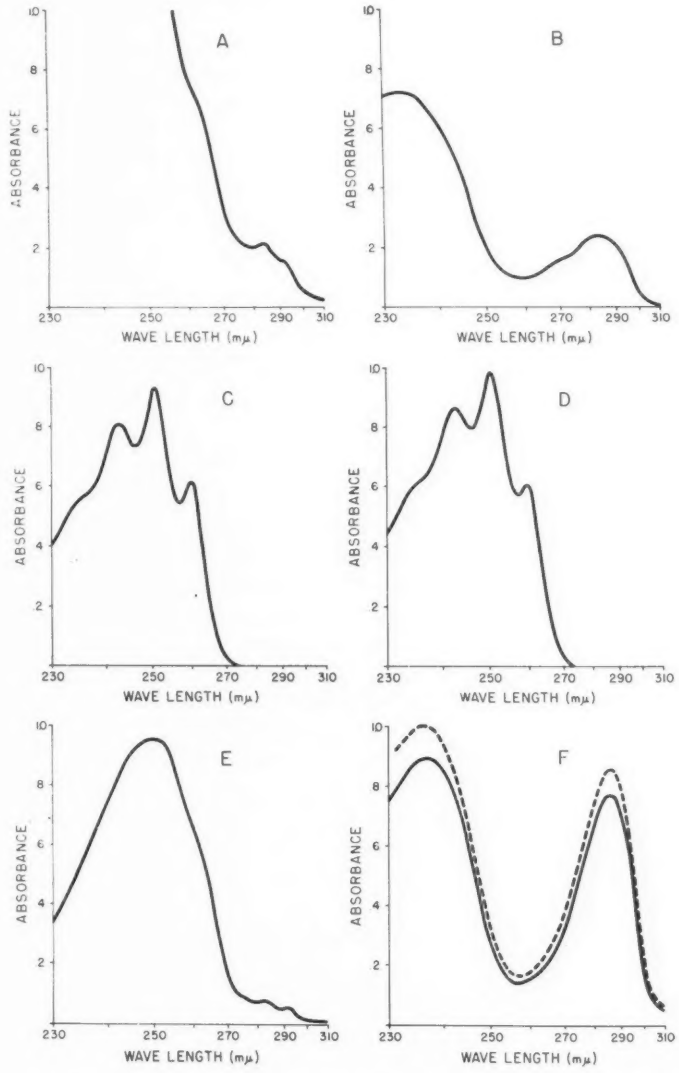


FIG. 1 Ultraviolet absorption spectra of (A) crude Hytakerol®; (B) sesame oil USP; (C) crystalline dihydrotachysterol; (D) chromatographically separated spot I from Hytakerol®; (E) spot III from Hytakerol®; (F) spot II from Hytakerol® (solid line) and from sesame oil U.S.P. (broken line). Spectra were taken in methanol except for (A) and (B) which were in dichloromethane. Concentrations were appropriately adjusted to show spectral details.

Spot II gave two peaks at 236 and 286 mμ of relatively low absorbance (spectrum F, solid line). A similar spot was obtained when sesame oil USP was chromatographed (spectrum F, broken line), indicating that this material was a contaminant in the sesame oil preparation.

Spot III absorbed strongly at 250 mμ with lesser peaks at 283 and 291 mμ (spectrum E). This compound did not reduce blue tetrazolium and gave a negative sodium hydroxide fluorescence. Spot tests suggested that this compound was some form of a peroxide. Ultraviolet irradiation causes a rapid decrease in its absorbance.

Chemical Assay

Since our biological results suggested that Hytakerol was less potent when compared with crystalline dihydrotachysterol on a milligram for milligram basis, several different lots of Hytakerol were assayed for "dihydrotachysterol" content.

Ten μg of Hytakerol from each lot were chromatographed and spot I (the material from Hytakerol exhibiting a dihydrotachysterol-like spectrum) was eluted as described above. Optical densities were determined at 250.5 mμ in a manual Beckman DU spectrophotometer and a spectrum of the same solution was made on the DK-2 recording spectrophotometer. A standard solution of 1.25 mg. of crystalline dihydrotachysterol in sesame oil U.S.P. was similarly assayed. Concentrations of the Hytakerol sterol were calculated from the absorbance values determined for crystalline dihydrotachysterol in methanol. Table 2 shows these results. It can be seen that all Hytakerol lots tested contained significantly more dihydrotachysterol-like ultraviolet-absorbing sterol (except for one lot, almost three times more) than the label indicated. The assay of a standard sesame oil solution of crystalline dihydrotachysterol, also shown in Table 2, demonstrates the reliability of the procedures utilized. Recovery approximated 100%.

TABLE 2. RESULTS OF CHROMATOGRAPHIC ASSAY OF HYTAKEROL STEROL

	Labeled* (mg./ml.)	Chromato. assay** (mg./ml.)	Chromato. assay / Labeled assay
Lot 303 RN	1.25	3.75	3.0
Lot 406 RC	1.25	3.57	2.9
Lot 060 SD	1.25	2.40	1.9
Lot 257 SD	1.25	3.40	2.7
Lot 281 SJ	1.25	3.47	2.8
Dihydrotachysterol in sesame oil	1.25***	1.24	1.0

* Bioassay determination by manufacturer. Label states that "1 cc. contains the equivalent of 1.25 mg. crystalline dihydrotachysterol."

** Chromatographic separation of Hytakerol sterol with concentration determination based upon molar absorbance of crystalline dihydrotachysterol.

*** Prepared by authors using crystalline dihydrotachysterol.

Mixed Chromatography

Five mg. of crystalline dihydrotachysterol were dissolved in 2 ml. of Hytakerol and 10 μg of this solution was streaked onto one-half of a 7 inch wide filter paper. A similar volume of the Hytakerol itself was streaked on the other half. The ends of the paper were serrated and the solvent front was allowed to run off the ends of the paper to obtain maximum separation of the compounds.

In addition to the three spots usually obtained with Hytakerol (Table 1) on all systems utilized, a fourth spot could be clearly identified only on the side of the chromatogram containing the Hytakerol with added crystalline dihydrotachysterol. This spot ran just ahead of spot I, the compound in Hytakerol® which exhibits the dihydrotachysterol-like ultraviolet absorption spectrum. When this fourth spot was eluted and its ultraviolet spectrum determined, a typical dihydrotachysterol spectrum was also obtained.

Infra-red Analysis

Separation of the Hytakerol sterol in a form pure enough for infra-red analysis presented many difficulties. Although we were not able to accomplish this separation completely, a pure enough preparation could be obtained for this analysis by first extracting 1 ml. of Hytakerol with 20 ml. of 60% methanol[6] and re-extracting the Hytakerol layer with 100% methanol. The 100% methanol extract was saponified with potassium hydroxide, extracted with petroleum ether, dried and redissolved in dichlormethane. A KBr pellet was then prepared from the dichlormethane solution. Figure 2 shows the infra-red spectra of the Hytakerol sterol (spectrum B) and our standard crystalline dihydrotachysterol (spectrum A). Although the peaks are not as prominent in the material obtained from Hytakerol, it can be seen that their locations are entirely different from those in crystalline dihydrotachysterol. These spectra are very similar to those published by van de Vliervoet and associates (13) for dihydrovitamin D_2II and dihydrotachysterol$_2$, respectively, although these authors only show the spectrum between 1300 and 700 cm^{-1}.

DISCUSSION

Although crystalline dihydrotachysterol appears to be the most potent "calcemic" sterol available (14, 15, 9), commercial preparations of "AT-10" employed clinically since 1933 (16, 7) have consisted primarily of relatively crude oil solutions labeled as containing "dihydrotachysterol." Originally, these preparations were made by reducing "tachysterol" with sodium and alcohol (7). Other reduction products (i.e., the dihydrovitamins D discussed below) were also known to be present and thought to possess

[6] This procedure was found to remove all of the "peroxide-like" material in Hytakerol which runs as Spot III.

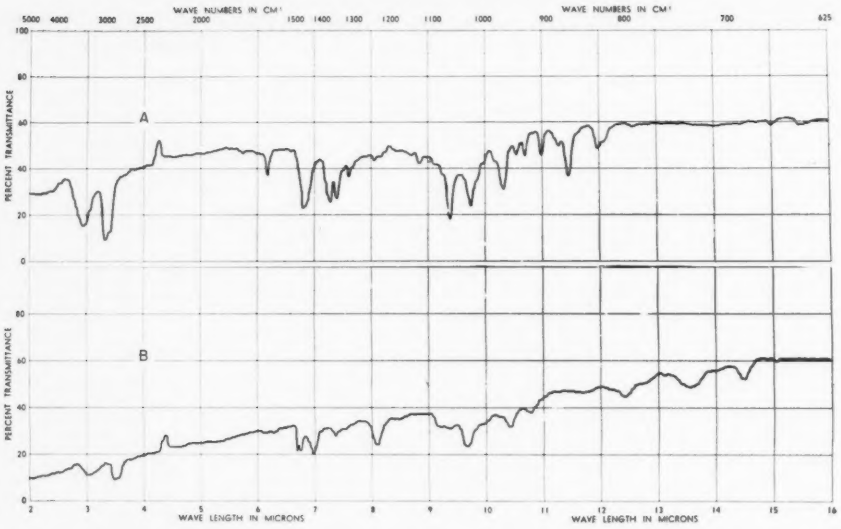


Fig. 2. Infra-red spectra of (A) crystalline dihydrotachysterol and (B) the dihydrotachysterol-like ultraviolet absorbing material separated from Hytakerol.

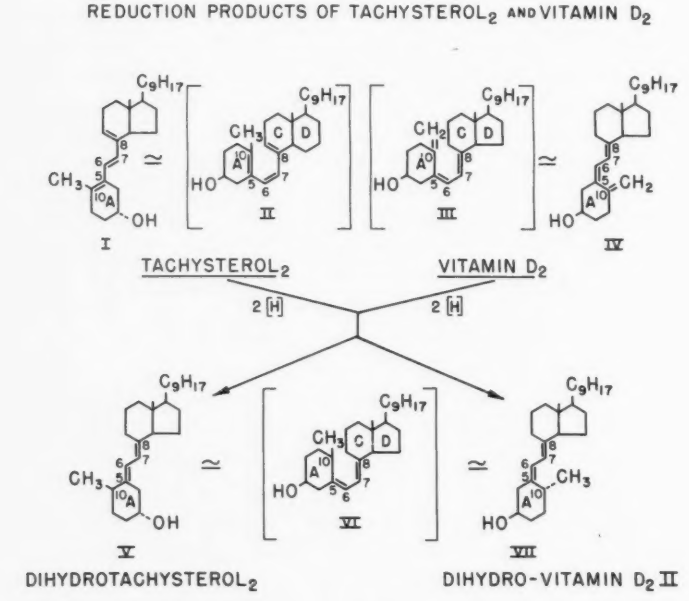


FIG. 3 Reduction products of tachysterol$_2$ and vitamin D$_2$. Classical formulas are shown in brackets. Note particularly the difference in attachment of ring A to the remainder of the molecule in the more stereochemically correct representations.

little or no calcium mobilizing activity (7, 12). For this reason the biological potency of commercial preparations was, and has continued to be estimated by bioassay of the crude mixture, rather than by chemical analysis of a separated and purified single active compound.[7] Thus the potency refers only to the hypercalcemic activity of the preparation (in rats) equivalent to a given weight of pure dihydrotachysterol used as a standard (17).

Our experimental results indicate clearly that the calcium mobilizing factor in the one such preparation marketed in this country (Hytakerol) is *not* dihydrotachysterol although it has a remarkably similar ultraviolet absorption spectrum. Chromatographically the Hytakerol sterol is less polar than dihydrotachysterol and its infra-red spectrum appears to be entirely different. These surprising facts led us to consider the possible identity of the active ingredient(s) in this preparation.

A clue as to its identity becomes apparent upon more detailed consideration of the chemical nature of the reduction process. Dihydrotachysterol can be produced in varying yields by reducing tachysterol, calciferol or even crude mixtures resulting from ultraviolet irradiation of ergosterol (7, 12). In each case, however, isomeric dihydrovitamin D_2 derivatives may be produced as illustrated in Figure 3, where the classical formulas (18) are represented in brackets (formulas II, III and VI) along with the more stereochemically correct representations (19). The characteristic relationship of ring A (formulas I, V, IV and VII) to the remainder of the molecule apparently determines whether a dihydro derivative is termed a dihydro*tachysterol* or a dihydro*vitamin D*, since both compounds are formed, but to varying degrees, during reduction of either tachysterol or calciferol (7, 12, 15, 20). While only dihydrovitamin D_2, isomer II, is illustrated in Figure 3 (formula VII) five others (Fig. 4) have been isolated and at least partially characterized (15, 20).

In view of the preceding discussion it would appear that the most likely hypercalcemic material presently in Hytakerol is one (or more) of the dihydrovitamin D_2 isomers. Since the net ultraviolet absorption spectrum closely resembles dihydrotachysterol, this would limit the predominant sterol to either isomer D_2II or D_2IV (Fig. 4) as only these have the necessary conjugated double bond structure. The D_2V and D_2VI isomers show relatively strong peaks at 244 and 246.5 mμ respectively (20) and therefore could only be present in trace amounts, if at all. Isomer D_2I, on the other hand, possesses an unconjugated double bond system and consequently has no ultraviolet absorption at wave length higher than 240 mμ (7). On the basis of the extremely low hypercalcemic potency of D_2IV,[8] it would

[7] In view of the fact that no commercial preparation heretofore available has utilized pure crystalline dihydrotachysterol, it seems advisable to refer to the commercial products by the general historical term "AT-10" or by specific brand name, e.g., Hytakerol. Dihydrotachysterol should be used only when referring to the pure compound.

[8] It has been reported that the potencies, relative to dihydrotachysterol$_2$, of dihydrovitamins D_2II, IV, V, and VI are 0.2, 0.01–0.02, 0.6 and 0.4 respectively (20).

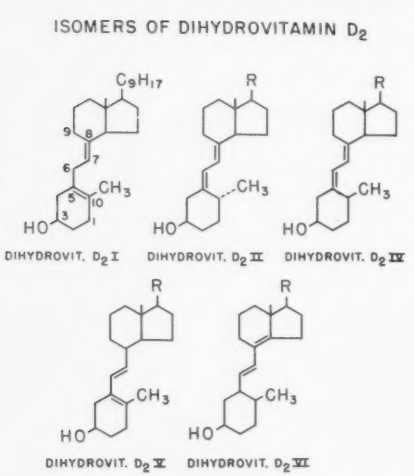


FIG. 4. Suggested stereochemical structures of the various dihydrovitamin D_2 isomers reported in the literature. Isomer III has not been sufficiently characterized.

seem that a major fraction of the hypercalcemic activity of Hytakerol is due to dihydrovitamin D_2II, although the presence of other dihydro derivatives cannot be excluded on the basis of the paper chromatographic separations alone.

It is not clear how the manufacturing processes for commercially produced AT-10 have evolved through the years, but if dihydrotachysterol were indeed the major constituent of the original AT-10 preparations, manufacturing processes must have been altered considerably since that time. Of interest in this regard is the statement by Albright and Reifenstein in 1948 (21) that AT-10 "is available in oil containing 1.25 mg. per cc. Prior to June, 1942, the same preparation was labeled 5 mg. per cc. because of the presence of then unrecognized inert materials."

Although 1 ml. of Hytakerol is labeled as being equivalent in hypercalcemic activity to 1.25 mg. of dihydrotachysterol, our studies (10) indicate that it is in fact less potent. In view of the greater content of ultraviolet absorbing sterol per ml. (Table 2), we would estimate that on a milligram for milligram basis the Hytakerol sterol is on the order of $\frac{1}{5}$ as potent as crystalline dihydrotachysterol, in agreement with the reported estimates of the potency of dihydrovitamin D_2 II shown in footnote 8.

Four of the five lots of Hytakerol which we tested contained 2.7 to 3.0 times the labelled quantity (1.25 mg./ml.) of "dihydrotachysterol" (Table 2); one lot was significantly less (by $\frac{1}{3}$) than the others (Lot 060-SD). Thus, variability in clinical potency between different lots would also be expected. As crystalline dihydrotachysterol$_2$ is now available, it would seem more desirable to utilize this material, if indicated for clinical management, in preference to previously available commercial preparations.

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PLASMA LEVELS OF "FREE" PROGESTIN DURING THE ESTROUS CYCLE IN THE MOUSE[1]

IRVING GUTTENBERG

Department of Anatomy, Yale University, New Haven, Connecticut

ABSTRACT

Twenty-four regularly cycling female CHI mice were exsanguinated at known points in the estrous cycle (as determined by vaginal smear). Their reproductive tracts were removed and examined histologically to confirm the phase of the cycle. Plasma samples from each animal were bioassayed for "free" progestin, and the results, expressed in Progesterone Equivalents (P.E.), were examined in relation to the phases of the cycle. It was noted that the plasma progestin content is at a minimum (0.5 P.E./ml.) in diestrus, rises to a maximum during proestrus and estrus (average about 4 P.E./ml.), and then decreases in metestrus-1 to approach the minimum in metestrus-2. The mouse is thus added to the list of animals secreting progestin before ovulation.

SINCE the development of the Hooker-Forbes bio-assay (1) cyclic variations in plasma progestin levels have been studied in the hen (2), rabbit (3), hamster (4), monkey (5, 6), and woman (7, 8, 9). These studies demonstrated the presence of progestin in the blood prior to corpus luteum formation. The purpose of the present study is to determine the peripheral blood plasma levels of "free" progestin during the various phases of the estrous cycle of the mouse, with particular attention to the preovulatory part of the cycle. Before this study it was not known whether the mouse secretes progestin prior to ovulation.

MATERIALS AND METHODS

The estrous cycles of 101 female mice, aged 75 to 105 days, of the highly inbred CHI strain were followed by means of wet vaginal smears through a minimum of three cycles to determine regularity of cycle. Of the 101 mice, 26 whose cycles showed a high degree of regularity were killed by exsanguination at various phases of the cycle (as determined by vaginal smear). Their reproductive tracts were immediately removed and fixed in Lavdowsky's fluid. Later, an ovary, the uterus, and the vagina were imbedded in paraffin, sectioned serially at 6 μ, stained, and examined microscopically to confirm the phase of the cycle.

The exsanguination procedures were performed between 10 A.M. and 2 P.M. by dividing the carotid sheath with the mouse under light ether anesthesia. The draining blood

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(0.5 to 1.0 ml.) was collected in a small centrifuge tube containing 0.05 ml. of a 20% sodium citrate anticoagulant solution and was centrifuged for five minutes. The plasma was then removed by a syringe and added drop by drop to 10 ml. of acetone at 6° C, thereby separating the "free" (acetone-soluble) and "bound" fractions of progestin. The tubes were stored at 6° C, and at a later time their contents were bio-assayed for "free" progestin by the method of Hooker and Forbes (1).

RESULTS

Of the 26 plasma samples obtained for this study, two had to be discarded because of technical difficulties.

Using the criteria described by E. Allen (10), histological examination of the reproductive tracts of the remaining 24 mice definitely established that five mice were in diestrus when their blood samples were obtained; eight, in proestrus; four, in estrus; two, in metestrus-1; and five, in metestrus-2.

The bio-assay results (Fig. 1) are expressed in terms of Progesterone Equivalents (P.E.). One P.E. is equal to the *activity* of one microgram of progesterone when assayed by the Hooker-Forbes method in the absence of estrogen or other interfering factors (10). The levels of "free" progestin found in this study ranged from 9.1 to less than 0.5 P.E./ml. plasma. No attempt was made to define subdivisions of each phase of the cycle, all

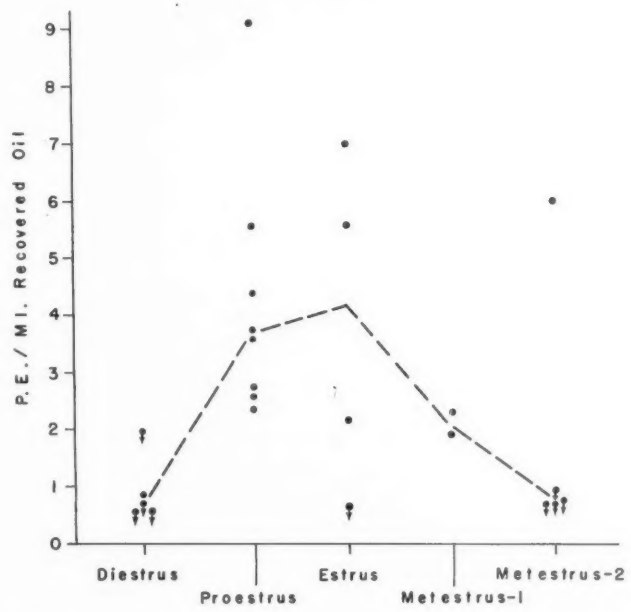

FIG. 1. Plasma progestin levels during the estrous cycle of the mouse. The dot and arrow symbol means that less than the indicated level of hormone was present. The broken line connects the median values for the phases.

samples for each phase being grouped together on the time scale of the graph.

When median values for each phase of the estrous cycle are compared, it appears that progestin secretion is minimal during diestrus, rises to a maximum during proestrus, is maintained through estrus, and then decreases in metestrus-1 to approach the minimum in metestrus-2 (Fig. 1). (The single low value at estrus and the single very high value during metestrus-2 could not be explained by examination of the ovaries or the course of previous estrous cycles, and were ascribed to technical error.)

DISCUSSION

The range of values observed during the complete cycle in this study (9.1 to less than 0.5 P.E./ml. plasma) is comparable to that found by Hooker and Forbes in a study of plasma progestin levels during pregnancy in the mouse (approximately 8.5 to less than 0.5 P.E./ml. plasma) (12). The values found during the preovulatory phase of the mouse cycle are, however, much greater than those which have been observed in the same phase in other species where preovulatory progestin has been detected. While the values ranged from 9.1 to 2.4 P.E./ml. during proestrus in the mouse, maximal preovulatory levels (based on relatively few determinations) in women were about 2.0 P.E./ml. (7); in monkeys, 1 or 2 P.E. (5, 6); in rabbits, about 3 P.E. (3); in hamsters, 1.2 P.E. (4). The explanation for this difference is not known.

The detection of significant levels of "free" plasma progestin during the preovulatory phase of the cycle in the mouse is not an entirely surprising finding in view of the evidence which has been accumulated for other mammals over the past thirty years. Observations on uterine contractility in the rabbit (13), on the mating behavior of the guinea pig (14), and of the rat (15), the classical study of the changes in the water content of the rat's uterus (16), and the induction of ovulation with progesterone in persistent-estrus rats (17,18) provided strong physiological evidence for the preovulatory secretion of progesterone. Significant levels of progestin have since been found by bio-assay in the plasma of the preovulatory hen (2), monkey (5, 6), rabbit (3), hamster (4), and woman (7, 8, 9).

The source of the plasma progestin detected prior to corpus luteum formation is uncertain, but appears to be the Graafian follicle. Corner has described luteal-like changes in the theca interna cells of the mature follicle of the sow (19). Dawson and Friedgood (20) suggested the granulosa of the preovulatory follicle of the cat as a source of progesterone. Subsequently, scattered luteal cells were observed in the walls of the follicles of several species including the mouse (10). Edgar, using a physico-chemical assay method, detected significant levels of progesterone in the Graafian follicles of the cow and the sow, and in a vein draining the ovary of the ewe, prior to ovulation (21, 22). Zander has isolated the hormone from the mature

human ovarian follicle (23). It is possible that in the mouse progesterone is produced by a non-ovarian source, but the fact that blood from the ovariectomized mouse gives a negative bio-assay response (1) argues against this.

The exact nature of the hormone assessed by the bio-assay is unknown. Edgar *et al.* have shown that the bio-assay can detect a minimal level of progestin in blood when chemical analysis cannot detect progesterone (24). Although specificity tests demonstrated that the bio-assay is relatively insensitive to 32 compounds thought to be related to or contaminants of progesterone (1, 25, 26), two naturally occurring metabolites, Δ^4-3-ketopregnene-20α-ol, and Δ^4-3-ketopregnene-20β-ol, have been found to elicit positive bio-assay responses in low concentrations (27). Moreover, certain ratios of progesterone to estrogen in the sample being analyzed can have a synergistic or antagonistic effect on the sensitivity of the nuclear response (28, 29).

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A STUDY OF THE PARTURIENT UTERUS WITH THE MICROELECTRODE TECHNIQUE[1]

HIROSHI KURIYAMA AND ARPAD CSAPO
The Rockefeller Institute, New York, N. Y.

ABSTRACT

The membrane potential of the post-partum uterus (estrogen-dominated) measured in single cells which microelectrodes during prolonged recording of membrane activity is about 45–50 mV, whereas that of late pregnancy is about 55–60 mV. The post-partum uterus periodically discharges trains of action potentials of considerable regularity in contrast to the irregular train discharges of the late pregnant uterus. During and after parturition the membrane activity of the impaled cell is in synchrony with the mechanical response, recorded from the uterine strip as a whole, because activity readily spreads in all directions. In the late pregnant uterus propagation is suppressed and membrane and myoplasmic activity are asynchronous. When labor is delayed by progesterone treatment the electrical and mechanical properties of the uterus are that of late pregnancy. Electrical activity is often preceded by prepotentials or slow depolarization waves which lower the threshold of excitability. These non-propagating cyclic depolarizations are not generated at specific anatomical sites.

Oxytocin lowers the membrane potential of the parturient and post-partum uterus and potentiates the train discharge. Ca-deficiency depolarizes the membrane and suspends membrane activity. Oxytocin becomes ineffective. A trace of Ca (0.12–0.25 mM) slightly increases the membrane potential and restores the oxytocin response. However, under these conditions oxytocin hyperpolarizes the membrane.

In vitro progesterone treatment of the parturient or post-partum uterus suspends membrane and myoplasmic activity. The membrane is slightly hyperpolarized and the action potentials gradually diminish.

IN THIS study the membrane and myoplasmic activity of the uterus is correlated under a variety of endocrine conditions, ie. before, during and shortly after delivery using two independent methods, the intracellular microelectrode and the sucrose gap techniques. This paper reports the observations made with intracellular microelectrodes (1). Another article in this volume describes the experiments with the sucrose gap (2).

A persistent problem in the electrophysiological investigation of smooth muscle is the discrepancy between the theoretical and measured values of the membrane potentials. As pointed out (3) the calculated membrane potential of the estrogen-dominated uterus, computed from Horvath's (4)

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data on the [K] outside and inside of the myometrial cell, is about 82 mV, whereas the measured value is about 43–48 mV (5, 6) or 55 mV (7). Although the membrane potential of the progesterone-dominated uterus is some 10 mV greater, and one often records values as high as 70 mV, the discrepancy between the means of the measured potentials and the calculated value remains. This discrepancy is widely recognized by other investigators and all observers uniformly report low membrane potentials of large scatter in smooth muscles (1, 5–22).

It is important to determine whether the low membrane potential is a genuine property or an artifact due to the damaging effect of the penetrating microelectrode on the smooth muscle cell. Indeed one frequently observes a gradual fall in the membrane potential after penetration, and also a great scatter of values when a large number of cells are impaled. These symptoms of damage are implicated in some of the published reports. However in more recent work, when the microelectrodes were successfully kept inside myometrial cells for several minutes, the membrane potential of the resting cell was stable, although trains of action potentials induced strong mechanical activity periodically. In spite of the stability of the membrane potential during prolonged recording, the measured values were less than that calculated from the ratio $[K]_i/[K]_o$ (1, 5, 6, 7).

In the present article we do not attempt to explain low membrane potential values of the myometrial cell because our present experiments do not provide sufficient evidence for a firm conclusion. Certain possibilities will be discussed in the subsequent paper. We will present, however a number of records illustrating the stability of the membrane potential of the impaled cell during prolonged observation. If the penetrating microelectrode damages the cell membrane, this injury apparently does not increase with time since the resting potential remains at a constant level throughout the period of impalement. The initial injury, however, may reduce the membrane potential and if so the actual values may be higher than those recorded.

Thus, in spite of prolonged recording of the membrane potential and its apparent stability some uncertainties remain concerning its absolute value, and it appears rewarding to substantiate microelectrode observations on relative changes, at least, with an independent method. Bülbring and her colleagues (23) successfully applied both the microelectrode and sucrose gap techniques in experiments with the taenia coli of the guinea pig. This and the following article (24) report a series of observations employing these two methods using two strips of the same uterus. A quick pilot experiment was usually carried out on a third strip recording only isometric tension, in order to determine endocrine conditions, ionic effects, effective drug concentrations, etc. The results of these parallel experiments are in good agreement.

MATERIAL AND METHODS

The experiments were performed on uterine strips obtained from 65 Sprague-Dawley white rats and 30 New Zealand white rabbits. Out of these, 16 rats and 3 rabbits were used in the parallel experiments with the intracellular microelectrode and sucrose gap methods. The uteri of the remaining animals were studied by microelectrodes only. Typical examples were selected for illustration in this article from a large number of records, obtained after the successful penetration, with microelectrodes, of several hundred myometrial cells. Most of the observations were made on the post-partum uterus, whose spontaneous membrane activity is generally regular. In order to indicate the full spectrum of our observations, however, we will show certain types of irregularities in membrane activity.

The method of *excising* the uterus, the *mounting* of the strips in the experimental chamber, the preparation of the *microelectrodes* and our experimental *setup* has been described in a previous paper (6). In the present experiments we worked at a temperature of 35° C ± 1° C. Only one end of the uterine strip was fixed by pinning it on a paraffin block. The other end was connected to a Grass strain gauge (FT 02) whose output was displayed on one beam of a dual beam oscilloscope (Tektronix, type 502), the other beam being used for recording membrane activity.

The stage of gestation, hormone treatment and other variables will be described under *Results*. Figures were retouched to improve contrast for reproduction.

RESULTS

The relation between membrane and myoplasmic activity

It is well known from experiments on frog muscle (see for references (25, 26, 27)) that a single action potential triggers a twitch, a series of action potentials of low frequency gives rise to an incomplete tetanus, whereas action potentials of sufficient frequency initiate a smooth tetanus. It is also well established that the frequency of the action potential discharge and the duration of the train of action potentials are important because they determine the maintenance and duration respectively of the active state.

Our expeiments as well as those previously reported (7, 15, 20) show that there is a clear relation between action potential discharge and mechanical activity in uterine muscle. This observation, however, is seldom made because the spontaneously active uterus usually discharges repetitive action potentials rather than a single spike. Occasionally, however, it is possible to see, as illustrated by Figure 1, the twitch, incomplete tetanus and complete tetanus occurring in sequence. This record was obtained during the early phase of an oxytocin response to a threshold dose of the drug. The unstimulated parturient rabbit uterus used in this experiment is relatively quiescent during the first hours of exposure to Krebs solution *in vitro*.

Thus it appears that the mechanical activity can be graded from a twitch to a maximum tetanus by the frequency of the action potentials and by the duration of the train discharge. This relationship between membrane and myoplasmic activity is of special significance in spontaneously active smooth muscles, since factors affecting spontaneous membrane discharge can control contractile activity.

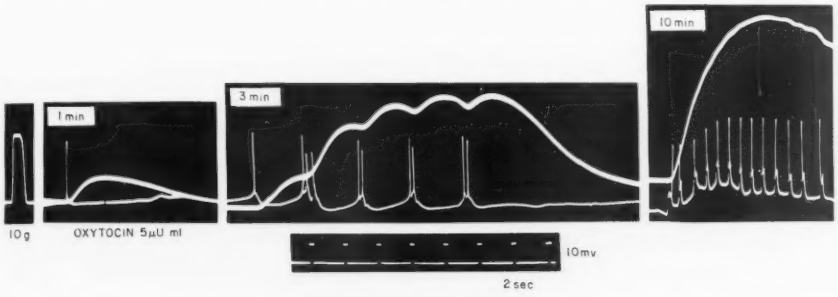

FIG. 1. Parturient rabbit uterus, treated with a threshold concentration of oxytocin. Recording is made during the initial phase of the muscle response. Note the relationship between action potential and mechanical activity.

Membrane activity in the post-partum rat uterus

In the rat uterus shortly after delivery several successive cycles of membrane and myoplasmic activity can be recorded without reimpalement. This uterus is especially suited for the study of spontaneous activity in that the action potentials are dischared in regular, frequent intervals and membrane activity is synchronous with myoplasmic activity.

Figure 2 is a record, illustrating a typical finding. The membrane potential is stable (at 50 mV) throughout the observation when the impaled cell

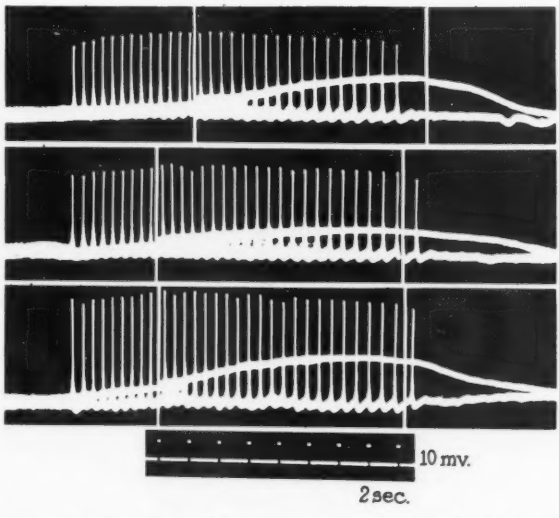

FIG. 2. Rat uterus, post-partum. Spontaneous membrane activity is recorded from one cell, without reimpalement, whereas mechanical activity is recorded from the whole uterine strip. Note the regularity of the train discharge, the stability of the membrane potential when the impaled cell is at rest and the synchrony between membrane and myoplasmic activity.

is at rest. The action potentials are regular having an amplitude of 40–50 mV and a frequency of about 1 per second. A train discharge consists of about 30 action potentials. The train appears at intervals of about 50 seconds and lasts for about 25 seconds.

The relative regularity of both membrane and myoplasmic activity and the synchrony of the two seen in this record is significant in view of the fact that membrane activity is recorded from one myometrial cell only, whereas the mechanical response from the whole strip. The synchrony is indicative of good propagation, whereas the stability of the membrane and the uniformity of the action potentials throughout the observation suggest that the initial damage resulting from impalement does not increase during the period of observation. It is, therefore of interest that the membrane potential in this condition averages only 50 mV. The post-partum rabbit uterus displays quite similar activity, which however is very infrequent for a period of about 12 hours after delivery (1).

Variations in the membrane activity of the post-partum rat uterus

Membrane activity of the post-partum uterus may show considerable variations (Table 1) not only from one animal to another but also in different portions of the same uterus. In our experiments with the rat and rabbit uterus we usually see three types of train discharges. One type is characteristically preceded by a slow depolarization wave, whereas in the second type each action potential in the train is preceded by a prepotential. In the third type we see train discharges without preceding slow waves or prepotentials. Figure 3 is a record illustrating characteristic variations in the prepotentials preceding the action potentials. These train discharges are recorded from three different cells of the same uterine strip. No marked

TABLE 1. ACTION POTENTIALS OF THE PARTURIENT AND POST-PARTUM RAT UTERUS

	No. of cases measured	Range	Mean	S.D.	S.E.
Amplitude mV	115	25 – 65	44	5.1	0.5
Duration (50%) msec.	30	27 – 75	47	16.6	3.1
Number in one train	45	13 –117	44	21.0	3.1
Frequency sec.	86	0.32– 1.8	0.73	0.27	0.02
Prepotentials of the Parturient and Post-partum Rat Uterus					
Amplitude mV	43	2 – 11	5.5	2.6	0.4
Duration sec.	43	0.08– 1.2	0.49	0.32	0.02
Slow Waves of the Parturient and Post-partum Rat Uterus					
Amplitude mV	9	5 – 13	7.5	2.8	0.9
Duration sec.	9	7 – 23	15	3.8	1.3
Membrane Potential of the Parturient and Post-partum Rat Uterus					
Magnitude mV	130	32 – 70	48	5.3	0.48

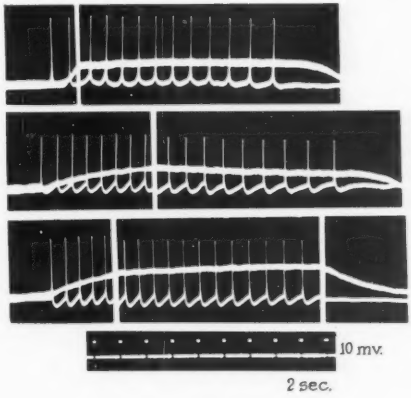

FIG. 3. Rat uterus, post-partum. Spontaneous membrane activity is recorded from three cells of the same uterine strip. Note the differences in the prepotentials.

prepotentials are seen in the action potentials discharged by the first cell whereas the action potentials of the second and third cells are preceded by distinct prepotentials.

The pacemaker area in heart muscle discharges action potentials with marked prepotentials (28, 29). But whereas in the heart the pacemaker area has a well defined anatomical location, in the uterus the cells discharging prepotentials are not limited to distinct anatomical sites. Our observations suggest that every myometrial cell is a potential pacemaker since we can record prepotentials from cells along the entire length of the uterine horn. It appears reasonable to assume that in the parturient uterus the cells which first recover from the progesterone block become pacemakers.

The magnitude of the prepotential has considerable variation (see Table 1). As will be discussed later, the prepotential may or may not give rise to a propagated action potential, since its effect depends on the threshold of the cell and perhaps on the time of its arrival during the refractory period of the cell membrane.

The prepotentials are to be distinguished from the "slow waves" described by Bozler (30). The slow depolarization waves preceding the train discharge have a distinctly different time course from that of the prepotentials. Figure 4 is a record, illustrating the characteristic slow depolarization waves (see also Table 1). It also illustrates that depolarization may continue after the first action potential is discharged and that this slow continued depolarization coincides with an increase in spike frequency and a decrease in spike amplitude. During the second half of the train the membrane and action potentials increase while the discharge frequency decreases. This type of train discharge is similar to that observed by Bülbring et al. (23) in the taenia coli of the guinea pig. We do not frequently record these slow depolarization waves in the myometrium.

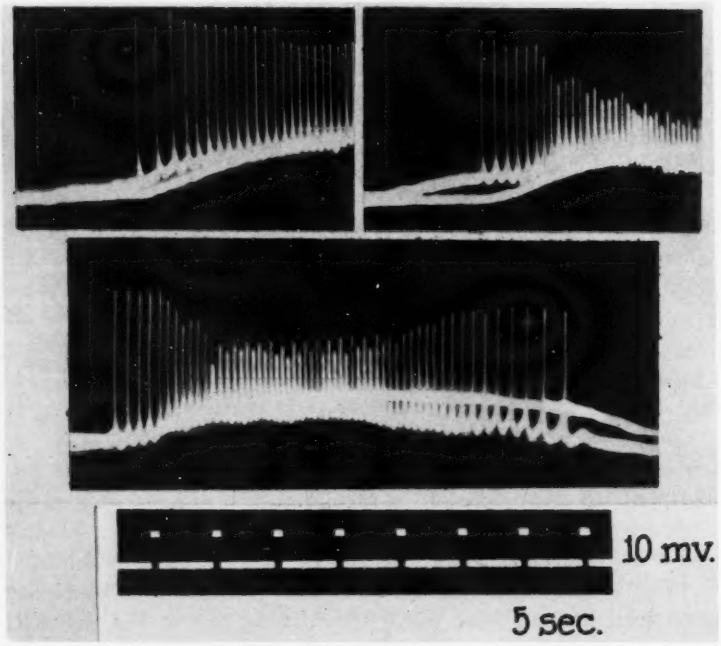

FIG. 4. Rat uterus, post-partum. Membrane activity is recorded from different cells at different areas of the same uterine strip. Note the slow depolarization waves preceding the trains of action potentials. Note also the inverse relation between frequency and spike amplitude, with depolarization and repolarization respectively.

We believe that the membrane activity recorded in these experiments is genuine. Figure 5 is a record illustrating a drop in membrane and action potentials almost certainly due to membrane damage or to dislocation of the electrode from the cell during membrane activity. We consider this type of change in membrane and action potentials an artifact.

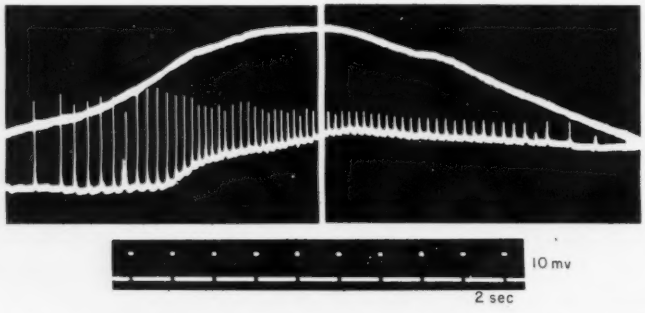

FIG. 5. Rat uterus, post-partum. A decrease in membrane and action potentials is illustrated during the recording of a train discharge. This type of sudden change is most likely an artifact.

Our present understanding of the nature and significance of the variations in membrane activity is inadequate. In the post-partum uterus we usually observe regular membrane activity, but occasionally the train discharge is irregular. Figure 6 illustrates typical irregularities when trains of action potentials seem to be composed of two train discharges superimposed on one another. There are alternate explanations for this kind of membrane activity. The action potentials may be generated in the impaled cell or may originate from adjacent cells. The change in spike amplitude may result from a change in the duration of the refractory period or from the timing of the spike in relation to the refractory period. It is also possible that a self-generated train becomes contaminated by the superimposed activity of neighboring cells. Our present experiments do not allow us to distinguish between these possibilities.

Our observations provide us with evidence for the spread of activity from one cell to the next, but they do not reveal the mechanism of propagation. We do not know with certainty whether the uterus is a structural or functional syncytium, and if the latter, whether conduction only involves electrical continuity or is mediated by way of a chemical transmitter. The problem of conduction in smooth muscle has been discussed by Sperelakis and Prosser (22).

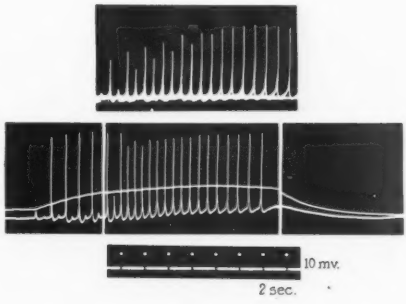

FIG. 6. Rat uterus, post-partum. Both records illustrate irregular trains of action potentials which seem to be composed of two superimposed train discharges. Note the gradual increase in spike height of the suppressed train discharge as activity continues. Note also the effect of frequency on spike height.

Goto, Kuriyama and Abe (31) found that the "velocity of propagation" of the action potential in the mouse uterus is 2–100 cm/sec. The velocity of spread depends on the distance between the stimulating and recording electrodes and the location of the electrodes, that is, whether they are in the same or in different fiber bundles.

The action potential

The shape and magnitude of the action potential in nerve and muscle has been well accounted for by the ionic theory of Hodgkin, Katz and Huxley (see for references 32). Special emphasis was put on the "overshoot," the short-lived reversal of the membrane potential, predicted by the ionic theory. The first claim of similar observations in myometrial cells

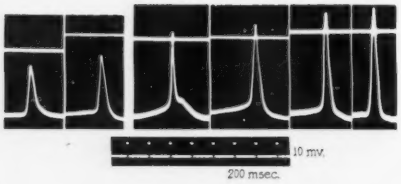

FIG. 7. Rat uterus, post-partum. The continuous horizontal line indicates zero potential. The action potentials are recorded from different cells of different uteri. Note the overshoot.

was made by Woodbury and McIntyre (13), and later by Jung (17), but these demonstrations were not fully convincing in view of the fact that overshoots were observed at membrane potentials as low as 30–40 mV. We seldom record overshoots in action potentials discharged by myometrial cells, and never at membrane potentials below 45 mV.

When the membrane potential is high and relatively stable, as is the case at the end of pregnancy in the rabbit, the myometrium is under partial block, and the action potentials are irregular and are not in synchrony with the mechanical response. It is the parturient or post-partum uterus which displays regular electrical activity well synchronized with the mechanical response. Action potentials from these uteri sometimes overshoot as illustrated by Figure 7. There seems to be a correlation between the magnitude of the potential and overshoot.

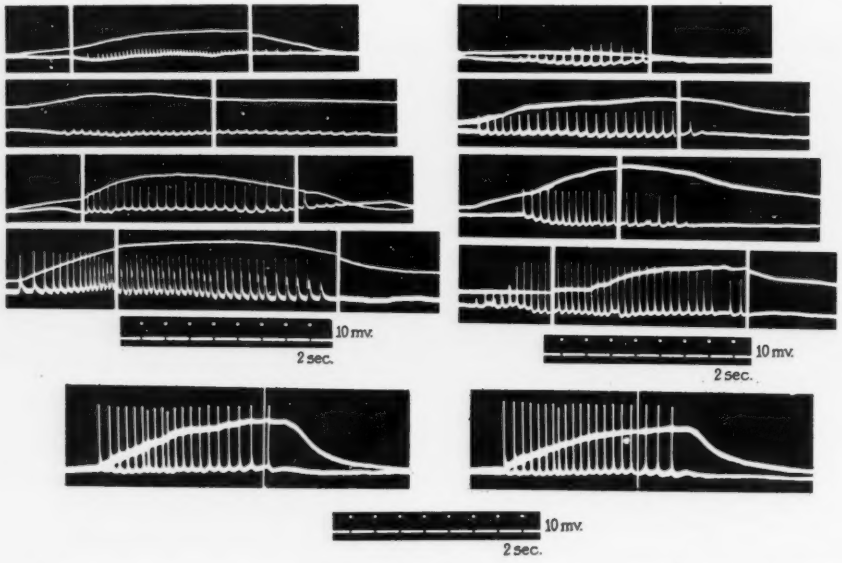

FIG. 8. Rat uterus. Upper left: 19th day of pregnancy; upper right: 23rd day of pregnancy, parturition being delayed by progesterone treatment (3 mg./day in oil, i.m.). Lower record: post-partum. Note the irregularity and small amplitude of the action potentials and the asynchrony between membrane and myoplasmic activity in the pregnant uteri as opposed to the post-partum uterus.

The progesterone-dominated uterus

Thus far we have described the characteristic membrane activity of the post-partum uterus. The uteri we have obtained during the short act of parturition showed spontaneous activity of essentially similar type, but less frequent. If the animal is sacrificed 48 hours before parturition or delivery is postponed by progesterone treatment for 24–48 hours a different type of membrane activity is observed. Figure 8 illustrates a set of typical records obtained in the uteri of such animals. The amplitude of the action potentials show great variation and very often only local potentials are recorded. The train of action potentials is not in synchrony with the mechanical response. If a train discharge is in synchrony with the mechanical response, which is seldom the case, the train is irregular in that the action potentials differ among themselves and are often of unusual shape. Such membrane activity gives rise to irregular mechanical activity as previously described (3, 33, 34, 35).

Progesterone blocks propagated membrane activity at a high membrane potential both in rats and rabbits (6, 7). The late pregnant rabbit uterus, like that of the rat, usually discharges irregular action potentials of small

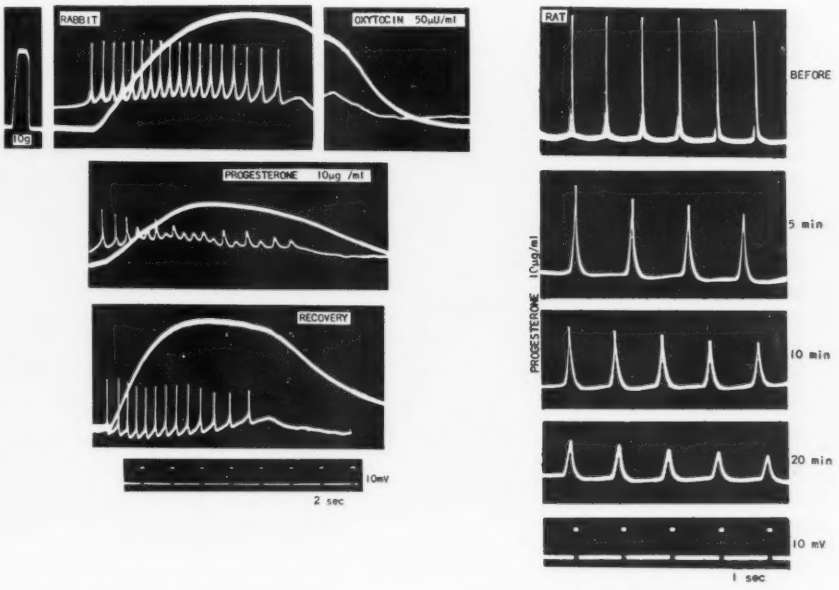

FIG. 9. Parturient rabbit and rat uterus. The records illustrate the *in vitro* effect of 10 μg/ml. progesterone on the mechanical and electrical activity of the uterus. The rabbit uterus is driven by oxytocin throughout the experiment, whereas the rat uterus is stimulated spontaneously. Note the decrease and return of activity on addition and withdrawal respectively of *in vitro* progesterone.

amplitude and the train discharges are not in synchrony with the mechanical response. After several hours of perfusion *in vitro* the action potentials improve slightly. The asynchrony between membrane and myoplasmic activity cannot be corrected by oxytocin.

The in vitro progesterone effect

Both spontaneous and oxytocin-induced activity of the parturient uterus are suppressed or suspended by *in vitro* treatment with 10 μg/ml. progesterone. The membrane becomes slightly hyperpolarized. The action potentials diminish in size until only local potentials remain as illustrated by Figure 9. Repeated rinsing with normal Krebs solution results in complete recovery of membrane and myoplasmic activity. This effect of progesterone on membrane activity explains the observation (33, 36) that *in vitro* progesterone treatment reduces or suspends the excitability and mechanical activity of the uterus. It is of interest to note that Schofield and Glenn (37) were unable to reproduce these effects of progesterone on the uterus *in situ*. Experiments using 4-C^{14} progesterone showed that *in situ* treatment could not approximate the progesterone concentration of the uterus treated *in vitro*. Furthermore, if the *in vitro* experiment was carried out in plasma rather than in Krebs, 15 times more progesterone was needed to obtain a comparable effect on excitability.

The oxytocin effect

The effect of oxytocin on the membrane and mechanical activity of the uterus has been previously studied (1, 12, 13, 20, 34, 38, 39). Apparently a prerequisite for the regulating effect of oxytocin is that the myometrial cell either is discharging regular trains of action potentials or is capable of doing so. If regular membrane activity is absent as is the case in the immature, in the progesterone-dominated, in the K depolarized, and in the "Ca-deficient" uterus, oxytocin is ineffective.

The characteristic oxytocin effects are an increase in the frequency and number of the action potentials as illustrated by Figure 10. The trains of action potentials also appear more frequently, increasing the repetition frequency of the mechanical activity. Threshold concentrations of oxytocin slightly depolarize the membrane without decreasing significantly the spike height. Higher concentrations reduce the magnitude of both membrane and action potentials markedly, and in extreme cases result in contractures. The effective oxytocin concentration is determined by the endocrine condition of the uterus (34).

When the membrane potential is at about 50 mV as is the case in the parturient uterus, the oxytocin effect (0.5–50 μU/ml.) often involves a periodic slight slow depolarization wave preceding the train discharge. If the membrane is depolarized by about 20 mV by Ca-deficiency, oxytocin is ineffective. But if the membrane potential of the Ca-deficient uterus is

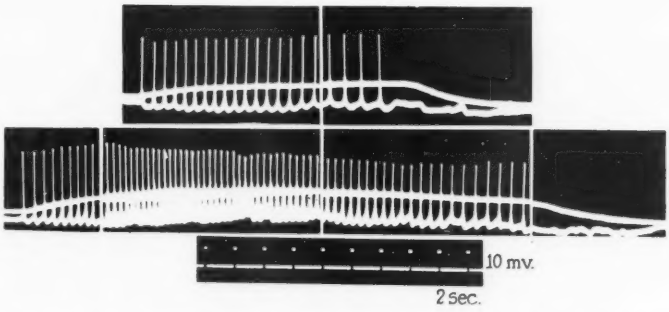

FIG. 10. Rat uterus, post-partum. Upper record: spontaneous activity; lower record: 50 μU/ml. oxytocin. Note the increase in the frequency and in the number of the action potentials.

slightly increased by partial replacement of Ca (1/10–1/20 of the normal Krebs Ca), oxytocin *increases* the membrane potential, spike height and frequency (Figure 11). It appears that oxytocin brings the membrane potential close to the "firing range" by a depolarizing or hyperpolarizing action respectively, depending on the level of the membrane potential before oxytocin treatment. The change of membrane potential and the subsequent effects in the train discharge, associated with oxytocin treatment, signal some significant but as yet unknown change in the physical properties of the excitable membrane. Participation of the Ca ion in this change is strongly implicated. An alteration in the physical state of the

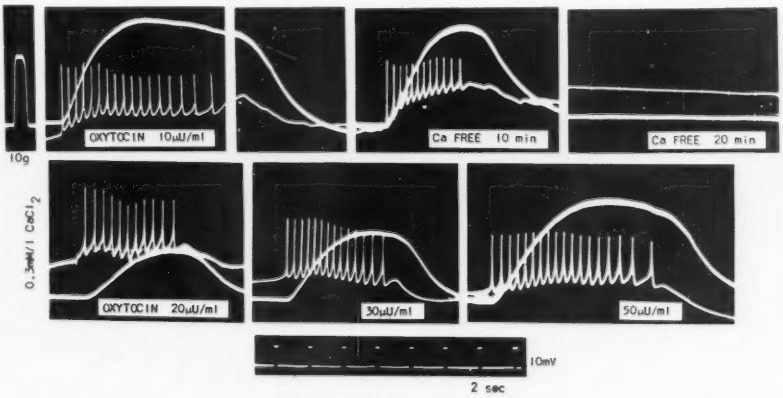

FIG. 11. Parturient rabbit uterus, driven by oxytocin. Upper row: the uterine strip is rinsed in Ca-free Krebs. Note the suspension of membrane and myoplasmic activity and also the drop in membrane potential. Lower row: 0.3 mM $CaCl_2$ is restored to the Krebs and the oxytocin concentration is increased in steps. Note the increase in membrane potential and the return of membrane and myoplasmic activity.

membrane could lead to permeability changes affecting the activity of the membrane (40).

DISCUSSION

The relationship between membrane and myoplasmic activity of the uterus is significant because it explains how spontaneous membrane activity can grade and thus control myometrial activity. Regulatory agents can set mechanical activity at any desired level by altering the frequency of the action potentials. In the progesterone-dominated uterus one records spikes with characteristically reduced amplitude. But this regulatory effect may result from disturbances in propagation, rather than from a primary effect on the action potential itself. By affecting the train discharge, regulatory agents can control function in smooth muscle organs, without the necessity of influencing the contractile system directly.

Progesterone dominance during pregnancy or in the non-pregnant animal is associated with a high membrane potential in the myometrium (6, 7) and a block of conduction (33, 34, 35, 41, 42). The post-partum uterus displays regular spontaneous membrane and myoplasmic activity, most likely as a result of the withdrawal of the progesterone block. As the membrane potential drops toward the "firing range," during and shortly after delivery, more and more cells become capable of discharging action potentials. Furthermore, the train discharge, initiated by any "pacemaker" cell is propagated to a considerable distance in all directions. All this facilitates synchronous membrane and myoplasmic activity.

The change in the properties of the excitable membrane of the myometrial cell prior to and during parturition is a singificant biological phenomenon far from being understood. Even during parturition the membrane potential is slightly above the "firing range" during rest and a prepotential or a "slow wave" must be generated by certain cells for triggering a train of action potentials. These electrotonic potential changes, however, are not necessarily mediated by the electrical activity of the impaled cell. The processes initiating these cyclic depolarizations and their anatomical location in uterine muscle are not known. Regulatory agents such as oxytocin, acetylcholine, serotonin, etc. are capable of inducing these changes (13, 20, 39, 40).

Jung (40) reported that oxytocin changes the permeability of the myometrial cell membrane, an effect which may not be the primary action of oxytocin but a consequence of it. If a uterine strip is rendered "inexcitable" by "Ca-deficiency," its membrane potential is reduced to 25–30 mV and it does not respond to oxytocin, but remains fully contractile (39). Ca concentrations of 0.12–0.25 mM increase the membrane potential slightly, and restore both excitability and pharmacological response to some extent. Oxytocin, under these conditions, hyperpolarizes rather than depolarizes the membrane before a train of action potentials is discharged (1, 2). This

effect of oxytocin is similar to the synaptic inhibitory neurone activity in crustacean stretch receptors described by Kuffler (43). In the stretch receptors the direction of the change in membrane potential, elicited by the inhibitory impulse, is determined by the initial level of the membrane potential. The primary effect of oxytocin is not known. It may constitute a physical change in the membrane in which the Ca ion participates. This participation of Ca in the regulation of membrane function is strongly implicated by the fact that oxytocin action depends entirely on the availability of membrane Ca.

Once the membrane potential in a cell or in a group of cells is reduced to a critical level, activity is generated (3), but during pregnancy the spread of this activity to the rest of the uterine tissue is inhibited by the progesterone block (33, 35, 42), so that an asynchrony between membrane and myoplasmic activity is set up. When the progesterone block is withdrawn propagation reappears in the uterus but how activity spreads from one cell to the other is not certain.

The regular membrane and myoplasmic activity of the post-partum uterus may be disturbed by too frequent spike discharge or by multiple "pacemaker" activity at different areas of the uterus. The active cell, from which action potentials are being recorded, may be influenced by self-generated or conducted discharges whose amplitude depends on changes in the duration of the refractory period or on the timing of the discharge in relation to the refractory period. If the spike frequency is too high the amplitude is reduced, a predictable effect from the known relation between membrane and action potential and spike frequency (23).

We have repeatedly recorded a series of train discharges from a given myometrial cell without reimpalement. The regularity of the action potentials in each of these trains, and the stability of the membrane potential between the periods of activity, suggests that the impaled cell may not be seriously damaged. The membrane potential under these conditions is not higher than previously reported and is similar to that obtained in the sucrose gap, as described in the subsequent paper (24).

We have seldom recorded action potentials with overshoot in spite of the successful penetrations of several hundred myometrial cells. When, during progesterone dominance, the membrane potential is high ($\cong$60 mV), the spontaneous discharges are usually small or irregular. When the membrane potential drops somewhat ($\sim$50 mV) during the withdrawal of the progesterone effect, overshoot may occasionally appear, but again disappears with regular activity at a membrane potential of about 45 mV.

Both the amplitude and duration of the action potentials show wide variations, especially between different uteri. At present we have no adequate explanation for these variations.

It is significant that the electrical and mechanical irregularity of the late pregnant uterus (progesterone-dominated) readily appears in the uteri of

animals whose delivery is delayed by progesterone treatment. The amplitude of the action potentials show great variations and very often only local potentials are recorded. Membrane and myoplasmic activity are not in synchrony due to the progesterone block. It is also interesting that the effect of progesterone in reducing the amplitude of the action potentials of the uterus can be demonstrated after *in vitro* treatment. A concentration of 10 μg/ml. progesterone slightly increases the membrane potential and reduces or eliminates the action potentials. The physiological significance of the *in vitro* progesterone effect is not clear, however.

The fact that two independent methods, the microelectrode and sucrose gap techniques, confirm each other in the results obtained is reassuring. The most significant points of agreement are stated in the subsequent paper. However, in comparison with the understanding of excitation in nerve, cross-striated and heart muscle, there is still much to be learned about smooth muscle.

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HORMONAL AND IONIC INFLUENCES ON THE MEMBRANE ACTIVITY OF UTERINE SMOOTH MUSCLE CELLS[1]

J. M. MARSHALL AND A. I. CSAPO

Department of Physiology, The Johns Hopkins University School of Medicine, The Rockefeller Institute and The Marine Biological Laboratory, Woods Hole, Massachusetts

ABSTRACT

Membrane resting and action potentials were recorded from strips of uterine muscles selected from rats and rabbits at known stages of pregnancy. The measurements were made with the "sucrose gap" methods which permits continuous recording of membrane activity for several hours.

Strips of muscle from a parturient, i.e. estrogen-dominated, uterus exhibited the following; (a) Cyclic periods of quiescence and activity, which were characterized by alternate depolarization and repolarization of the membrane. Action potentials were discharged during the depolarization phase. (b) Oxytocin, applied during a quiescent period, immediately depolarized the membrane and potentiated the action potential discharge. (c) Perfusion with Ca-free Krebs solution depolarized the membrane to a level where all action potentials were abolished and oxytocin was ineffective. (d) Addition of 0.25 mM Ca (10% of normal concentration) to the Ca-free solution repolarized the membrane somewhat and under these conditions oxytocin further increased the membrane potential to a level where action potentials were generated.

Strips of muscles from late pregnant animals, i.e. progesterone-dominated, showed no cyclic membrane activity and were unresponsive to oxytocin.

Isotonic potassium chloride depolarized both estrogen and progesterone-dominated strips about 28 mV. Istonic potassium sulfate produced 46 mV of depolarization in the estrogen muscles and 54 mV in the progesterone muscles.

MANY difficulties are encountered when intracellular microelectrodes are used to study the membrane activity of smooth muscle cells. Owing to the small size and inherent motility of these cells the electrodes tend to dislodge making continuous measurements troublesome. An external electrode method, the "sucrose gap" technique, has recently been devised (1, 2) whereby membrane activity can be observed over relatively long periods of time. It is assumed that in the sucrose gap the muscle behaves as a core conductor. If so, the membrane resting and action potentials can be measured with this technique provided the short-circuiting between the electrodes in negligible. This is accomplished by increasing

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the external resistance of the muscle between the electrodes by replacement of the interstitial fluid with sucrose solution.

The present paper reports the results of experiments employing the sucrose gap technique. An investigation was made of the effects, on the membrane activity of the uterus, of alterations in extracellular calcium and potassium ions and of oxytocin. A separate paper (3) will present observations obtained with intracellular micro-electrodes from single cells in strips of uterine tissue dissected from the same muscles used in the present study. It will be seen that there is a close correlation between the results of the two methods.

METHODS

Experiments were performed on the isolated uteri from three rabbits and sixteen rats selected at various stages of gestation. The progesterone-dominated uteri were obtained from rabbits between 20–29th day of pregnancy and from rats between the 15–18th day. Parturient and postpartum uteri were used for the estrogen-dominated specimens. Both species gave essentially the same results. In most experiments, one portion of the uterus was studied by the sucrose gap method and another portion by the intracellular microelectrode technique. Thus it was possible to compare the results of the extracellular and intracellular methods obtained from strips of the same muscle under identical conditions. The results of the intracellular method will be reported in another communication (3).

Longitudinal muscle strips were dissected from the uterus near its mesometrial attachment. In the progesterone-dominated group these pieces were cut from the areas of placental attachment, whereas in the estrogen group the interplacental regions were used. Strips about 3–5 cm. long and 0.5 mm. in diameter were carefully teased off from the longitudinal layer of these larger uterine pieces. One such strip was mounted in a sucrose gap apparatus, as described in detail by Burnstock and Straub (2). The middle portion of the strip lay horizontally in plastic tubing through which isotonic sucrose solution at about 12° C flowed continuously. Each end of the strip was pulled vertically upwards into plastic tubing through which Krebs solution flowed continuously.

The solution used in all experiments contained, mM; NaCl 118.46, KCl 4.74, $CaCl_2$ 2.54, $MgSO_4$ 1.18, KH_2PO_4 1.18, $NaHCO_3$ 24.87, dextrose 5.5. This solution was gassed with a mixture of 95% O_2—5% CO_2 throughout the experiment. The sucrose solution contained 10% (w/v) reagent grade sucrose dissolved in triply distilled water which had been passed over an ion exchange resin.

Krebs solution from a reservoir bottle, maintained at 12° C, flowed continuously down the verticle tubing containing one end of the muscle. This portion remained inactive, and thus maintained a stable potential against which the changes in potential at the "test" end could be compared. The opposite end of the muscle was bathed in either normal Krebs or test solutions at a temperature of 32–33° C. A two-way stop-cock was inserted into the line connecting the solution reservoirs with the test end of the muscle so that the tissue could be bathed in either normal Krebs or test solutions. The general procedure for all experiments was to bathe both ends of the muscle in normal solution for about thirty minutes and then to switch to the test solution at one end, leaving the reference end in normal Krebs.

The potential difference between the sucrose-Krebs junctions at either end of the muscle was recorded by a pair of cotton wick electrodes over a KCl-agar bridge to Ag-AgCl electrodes. The electrode at the test end of the muscle was connected, through a cathode follower, to a DC amplifier the output of which was displayed on a cathode

ray tube. The tube face was photographed on stationary film with a single frame 35 mm. camera. The electrode at the "reference" end of the muscle strip was connected to ground through a voltage calibrator. The polarity was so arranged that an upward deflection of the oscilloscope trace signaled negativety of the electrode at the test end.

In experiments involving *in vitro* oxytocin and progesterone treatment the compounds were added to the Krebs solution perfusing the test end of the muscle. In this way the tissue was continuously exposed to these substances. The isotonic KCl and K_2SO_4 solutions were 158 mM and 125.1 mM respectively. These solutions are isotonic on the basis of freezing point depression data.

RESULTS

Estrogen-dominated muscles

Normal activity

Five or ten minutes after the muscle had been perfused with normal solution at both ends, cyclic variations in membrane potential began to occur. These involved depolarizations at the test end of 5–10 mV accompanied by a discharge of action potentials. Subsequently repolarization of the membrane occurred and the muscle became quiescent. This activity lasted about 20 seconds and occurred once every 2–3 minutes. The records in Figure 1, obtained from a strip of parturient rat uterus, illustrate the fluctuations in membrane activity. These single frame photographs were

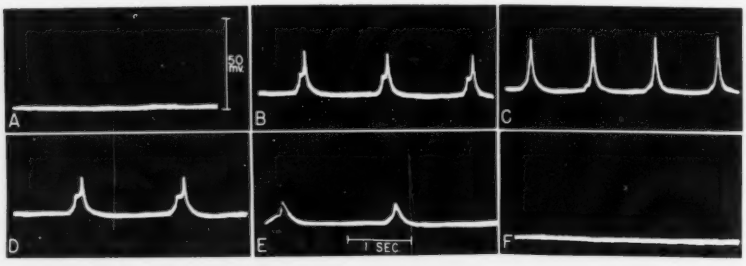

FIG. 1. Typical records obtained from a strip of estrogen-dominated rat uterus showing spontaneous membrane depolarization and action potential discharge. Single frame photographs taken at successive 2 second intervals illustrate transition from rest to activity and back again to the resting state. In this and all other records, an upward deflection of the oscilloscope trace indicates relative negativity, deplorization, of the "test" end of the muscle strip in the sucrose gap apparatus. Temp. 32° C.

taken at successive two second intervals. Figure 1, (A) shows the quiescent membrane. As the membrane depolorizes action potentials appear (B), and their rate of discharge increases as the membrane potential continues to decline (C). The membrane then repolarizes and the muscle returns to its resting state (D–F). The fluctuations in membrane activity were most probably associated with spontaneous contractions of the muscle. Simultaneous recordings of tension and intracellular action potentials from strips

of the same uterus under similar conditions showed contractions at about 2 minute intervals (3).

Action potentials recorded in the sucrose gap, showed considerable variation in both size and configuration. In general they were of lower amplitude than those obtained with the microelectrodes. This is to be expected, however, since the external electrodes are in contact with a number of fibers some of which may be firing asynchronously. The estrogen-dominated muscles gave the largest and most uniform action potentials, average value of 30 mV. It is known that in these muscles synchronous activity is typical (4).

Effects of oxytocin and "Ca-deficiency"

The addition of oxytocin (50 μU/ml.) to the solution bathing an inactive muscle caused an immediate depolarization of the membrane accompanied by a discharge of action potentials (Fig. 2, A–B). Oxytocin also doubled the frequency of the cyclic membrane activities.* On return to the normal

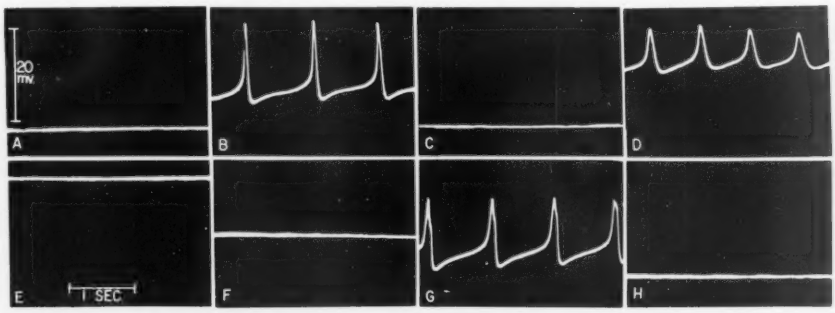

FIG. 2. Effects of oxytocin and low calcium solutions on membrane activity of the estrogen-dominated rat uterus. A. Quiescent period in normal Krebs. B. 1 min. after addition of oxytocin (50 μU/ml.). Note membrane depolarization and the appearance of action potentials. C. Return to normal Krebs. Between C and D perfusion switched to Ca-free solution, and record in D obtained 10 min. later in Ca-free solution. E. After 20 min. in Ca-free Krebs. F. 5 min. after addition of Ca (0.25 mM) to Krebs solution, note membrane has repolarized somewhat. G. 2 min. after oxytocin (50 μU/ml.) in presence of 0.25 mM. Ca. Note further *repolarization* of membrane by oxytocin. H. 25 min. later in normal Krebs. Temp. 32° C.

solution, the muscle regained its original rhythm of activity. If the same uterine strip was then bathed in a Ca-deficient solution, there was a gradual membrane depolarization. During this depolarization a transient period of activity appeared as the membrane potential passed through a critical region where action potentials were generated. After a period of about twenty minutes, the membrane of the test end had depolarized to a steady value of 20 mV negative to the reference end of the muscle, and at this

* This stimulatory action of oxytocin continued as long as oxytocin was present in the bathing solution.

point all activity ceased (C, D, E of Fig. 2). The addition of oxytocin in doses up to 100 mU/ml. was totally ineffective under these conditions. Calcium, 0.25 mM (10% of its concentration in normal Krebs), was then added to the bathing solution. This caused the membrane to repolarize towards its normal resting level by 8 mV (Fig. 2, F). The addition of oxtocin, 50 μU/ml., now caused a further increase of about 3 mV in the membrane potential and the appearance of action potentials (G). With a return to the normal solution the membrane potential regained its original control level (H).

The results of nine such experiments are summarized in Table 1. All of

TABLE 1. A COMPARISON OF THE EFFECTS OF OXYTOCIN ON MEMBRANE POLARIZATION OF CELLS FROM ESTROGEN-DOMINATED RAT UTERI (N-9) IN NORMAL KREBS AND IN Ca-DEFICIENT SOLUTIONS

	Oxytocin in normal Krebs (50 μU/ml.)	Ca-free Krebs	Oxytocin in Ca-free Krebs (100 mU/ml.)	0.25 mM Ca added to Ca-free Krebs	Oxytocin in 0.25 mM Ca (50 mU/ml.)
	Millivolts depolarization at test end				
Average	12	18	18	10	7
Range	(8–17)	(16–25)	(16–25)	(7–12)	(5–9)

these experiments were performed exactly as those described above. Under normal conditions oxytocin causes a depolarization of about 12 mV (average) in the estrogen-dominated uterus. Perfusion with calcium-free Krebs depolarized the muscles by 18 mV (average), partial replacement of the calcium (0.25 mM) repolarizes the membrane by 8 mV, the application of oxytocin now further *repolarizes* the membrane by 3. This brought the membrane potential into the critical range for the discharge of action potentials. It should be emphasized that oxytocin first repolarized the membrane and *then* action potentials appeared. Whether or not this action of oxytocin is specific only for Ca-depolarized membranes cannot be answered at this time. Future experiments with other depolarizing agents are planned.

It appears, then, that the action of oxytocin depends upon the magnitude of the membrane potential at the time of application. If the membrane is in its resting state, the stimulatory effect of oxytocin is associated with a *depolarization*, but if the membrane previously has been depolarized below the "critical" level for excitation, oxytocin *repolarizes* towards the critical level.

Effects of in vitro *progesterone*

In several experiments the muscle was first stimulated by oxytocin, and then progesterone (10 μg/ml.) was added to the test solution. About thirty minutes after the addition of the progesterone all action potentials had disappeared and the muscle was completely quiescent, even though oxyto-

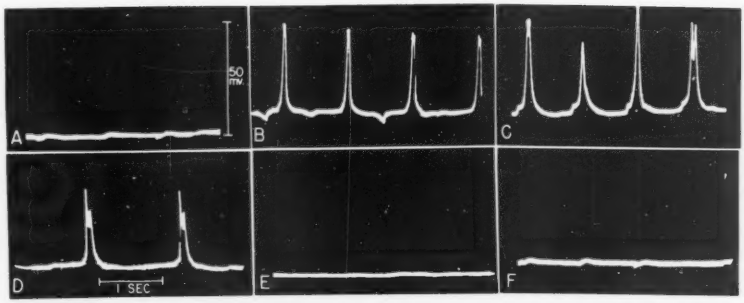

FIG. 3. Effect of *in vitro* progesterone on estrogen-dominated rat uterus. A. Resting muscle, normal Krebs. B. Several seconds after addition of oxytocin (50 μU/ml.) to the Krebs solution. Muscle now active, i.e. membrane has depolarized and action potentials appear. C. 10 minutes after the addition of progesterone (10 μg/ml.) to the bathing fluid. Note irregular form of action potentials. D. 20 minutes after progesterone. Action potentials smaller and their rate of discharge declining. E. 35 minutes later. All activity abolished. F. 50 minutes after return to normal Krebs. Membrane has returned to control level for quiescent muscle. Temp. 32° C.

cin was still present in the test solution. Records from one such experiment appear in Figure 3. The membrane activity of this muscle was undergoing the alternate periods of activity and rest as described previously. The record at A was taken during a resting period. At this time oxytocin was added and the strip was stimulated (B). Ten minutes after the additoin of progesterone to the bathing fluid, (Fig. 3, C), the contours of the action potentials have become irregular although their discharge rate remains the same. Twenty minutes later, however, the rate of discharge has declined, and the membrane potential is returning towards the control level. Thirty-five minutes later all activity has ceased, although oxytocin was still present in the test solution. At this time the membrane has hyperpolarized by about 3–5 mV as compared to the reference end (E). When the muscle was returned to normal Krebs, the membrane returned to its control level in about fifty minutes (F) and again began to undergo the spontaneous fluctuations, typical of an estrogen-dominated uterus.

Progesterone-dominated muscles

Strips taken from rat uteri between the 15–19th day of gestation, or from rabbit uteri between the 20–28th day of pregnancy showed no rhythmical fluctuations in membrane or action potentials within the first hour of observation. Furthermore they were totally unresponsive to oxytocin in doses uf to 20 mU/ml. Perfusion with solutions containing excess potassium or low calcium, however, caused membrane depolarization associated with abortive and irregular trains of action potentials. This activity was transient and only appeared as the membrane passed through a zone of

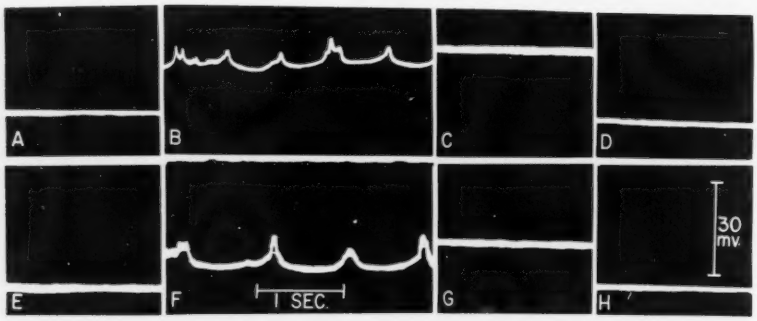

FIG. 4. Progesterone-dominated rat uterus. A-D. Effect of isotonic KCl. A. Krebs solution, no activity. B. 5 min. after introduction of isotonic KCl. Note abortive action potentials as membrane depolarizes. C. 15 min. in isotonic KCl. D. Return to Krebs. E-H. Effects of Ca-free solution. E. Krebs, no activity. F. 2 min. after introduction of Ca-free Krebs perfusion. G. 1 hour later in Ca-free. H. Return to Krebs. Temp. 32° C.

increased excitability. With further depolarization the muscle became inactive (Fig. 4).

Another difference between the progesterone-dominated and the estrogen-dominated muscles was found in their time course of depolarization in calcium-free solution. The estrogen-dominated muscles would depolarize by 18 mV to a steady level in an average time of 22 minutes, whereas the progesterone-dominated uteri would take an average of 81 minutes to reach approximately that level (Table 2).

One similarity between the progesterone and the estrogen-dominated uteri was their response to the depolarizing actions of isotonic KCl and

TABLE 2. A COMPARISON OF THE DEPOLARIZING EFFECTS OF Ca-FREE KREBS, ISOTONIC KCl AND ISOTONIC K_2SO_4 SOLUTIONS ON ESTROGEN-DOMINATED AND PROGESTERONE-DOMINATED UTERINE MUSCLE CELLS OF THE RAT, AND ON SKELETAL MUSCLE FIBERS OF THE RABBIT AND FROG

	Ca-free time for max. dep. in min.	mV depolarization at test end			
		Ca-free	Isotonic KCl	Isotonic K_2SO_4	Temp. ° C
Uterine muscle					
Estrogen (N-9)					
Average	22	18	28	46	32
Range	(16–28)	(16–25)	(24–30)	(40–54)	
Progesterone (N-9)					
Average	81	17	29	54	32
Range	(65–90)	(15–20)	(28–32)	(50–56)	
Skeletal muscle					
Rabbit (N-2)			68, 67	65, 68	32
Frog (N-5)					
Average			70	72	22
Range			(68–75)	(70–75)	

K_2SO_4. Both solutions produced a rapid depolarization which reached a steady level within 5–6 minutes. This depolarization was maintained for periods up to one and one half hours. The magnitude of depolarization in K_2SO_4 was always much greater than in the cholride, however (see Table 2). The values for sulphate depolarization approximated those of the resting potentials recorded with intracellular electrodes (4). It is interesting to note that the total depolarization was somewhat greater for progesterone-dominated uteri than for the estrogen-dominated uteri. This finding confirms earlier microelectrode observations (5, 6).

Several experiments were performed to compare the depolarizing effects of KCl and K_2SO_4 on striated and uterine smooth muscles respectively. Small bundles of fibers were carefully dissected from the leg muscles of several rabbits and frogs. The results, shown in Table 2, indicate that in striated muscle there was no appreciable difference between the magnitudes of depolarization in KCl and K_2SO_4.

DISCUSSION

The results indicate that the sucrose gap method used previously by Stämpfli (1) for isolated nerve fibers and by Burnstock and Straub (1958) for intestinal smooth muscle is also appliable to uterine smooth muscle. The theoretical considerations of this technique have been extensively described elsewhere (1, 2), and need not be detailed here. Several points, however, may be worth mentioning.

Uterine muscle cells, in contrast to skeletal muscle and peripheral nerve fibers, are only about 70–100 μ in length. Hence a single uterine cell does not extend from one electrode to the other across the sucrose gap. Assuming that the external resistance has been made extremely high, by replacement of the extracellular fluid with sucrose, the only connection between the electrodes is through some internal low resistance path. The fact that uterine muscle strips function in the gap indicates that some such pathway exists from cell to cell. The precise manner in which smooth muscle cells are connected is unknown, although suggestions of low impedance pathways (7), intercellular bridges (8), and even direct protoplasmic continuity (9), have been made.

A second consideration is that of junction potentials, and what effects if any, they may have on the potential difference recorded between the electrodes. The junction potentials arise at the following interfaces; 1) sucrose-Krebs on both sides of the gap, 2) Krebs-3M KCl wick electrode on the reference side, 3) Krebs-3M KCl wick electrode, 4) isotonic KCl-3M KCl wick electrode and 5) isotonic K_2SO_4-3M KCl wick electrode on the test side. Lacking detailed information about the short-circuiting factor (10), we cannot evaluate the sucrose-Kreb potentials. The values for the other potentials mentioned above have been calculated using the Henderson equation and the ionic mobilities suggested by MacInnes

(11). The Krebs-wick electrode junction gave 1.92 mv, electrode side positive. This increased to 7.4 mv when the Krebs solution was replaced by isotonic KCl and to 9.2 mv with isotonic K_2SO_4, all with the electrode side positive. When Krebs solution perfused both sides of the muscle strip, the junction potentials around the circuit balanced out and no potential difference was recorded between the electrodes. When the isotonic potassium solutions were applied to the test side the depolarization was much greater than could be attributed to junction potentials alone. Furthermore, the concentration of oxytocin was too small to alter the junction potential, but it caused a 12 mV depolarization of the test end. Also, Ca-free solutions depolarized the test end by 18 mV, although this solution does not alter junction potentials appreciably.

The magnitude of the potential difference recorded between the electrodes will depend, among other things, on the completeness of replacement of intersititial fluid with sucrose and on the amount of cell damage within the muscle strip. Incomplete washout with sucrose, i.e. a high external short circuiting factor, and an excessive amount of fiber damage will tend to lower the measured values. For these reasons it is best to consider the measured potential difference not as an absolute value for the membrane potential, but to consider it only as a relative potential change under various experimental conditions.

Depolarizations in isotonic K_2SO_4 greater than those observed in isotonic KCl agree with the findings of Burnstock and Straub (2) for intestinal smooth muscle and those of Schmidt and Huber (12) for guinea-pig uterus. Skeletal muscle, on the other hand, showed no such discrepancy in agreement with the data of Adrian (13). Recently, however, Hodgkin and Horowicz (14) found that a sudden change in chloride concentration, at constant potassium concentration, produced a transitory alteration in the membrane potential with frog sartorius, in the direction expected for a chloride electrode. Possibly in smooth muscle cells the chloride ion is of considerable importance in maintaining the resting potential, and for this reason only in isotonic K_2SO_4 would the extent of depolarization approach the resting potential value recorded with intracellular micro-electrodes.

In the estrogen-dominated uterus oxytocin causes either a depolarization or a repolarization depending upon the level of the membrane potential at the time of application. Under normal conditions oxytocin depolarizes the membrane and initiates a train of action potentials. If the membrane is depolarized, however, by continuous perfusion in Ca-free solution, to a level where all propagated activity ceases oxytocin is ineffective. When the membrane is depolarized just *slightly* below the "critical" level for excitation, oxytocin *repolarizes* the membrane bringing it into the zone where action potentials are discharged.

This action of oxytocin is reminiscent of the synaptic inhibitory neuron activity in the crustacean stretch receptor, described by Kuffler and his

associates (15). In this case an inhibitory impulse *repolarizes* a cell whose resting potential has been previously reduced; whereas when the cell is initially hyperpolarized the inhibition is associated with a *depolarization* of the membrane. An "inhibitory equilibrium level" is postualted, towards which the inhibitory impulse tends to drive the membrane potential. Perhaps the action of oxytocin is somewhat similar, tending to drive the membrane towards an "excitatory equilibrium level" when it is slightly displaced to either side of this level.

No such "excitatory equilibrium level" seems to exist in the progesterone-dominated uterus, since progesterone exerts a stabilizing effect on the membrane (5, 6) and renders it inexcitable to oxytocin. Furthermore, progesterone-dominated muscles appear to bind calcium more firmly than do the estrogen-dominated uteri as indicated by the observation that the former require a very much longer period of time for the depolarizing effects of low calcium to appear (Table 2; (5, 16). It is interesting to note that progesterone when applied *in vitro* gradually abolishes all spontaneous activity and leaves the muscle refractory to the action of oxytocin.

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FRACTIONATION OF PLASMA PARATHYROID HORMONE ACTIVITY[1,2]

LEO E. REICHERT, JR.[3,4] AND MAURICE V. L'HEUREUX

Department of Biochemistry, Stritch School of Medicine and the Graduate School, Loyola University, Chicago, Illinois

ABSTRACT

Components of rat and human plasma obtained by ammonium sulfate and cold ethanol fractionation procedures have been assayed for their calcium-mobilizing activity in parathyroidectomized rats. The determinations were made on fractions of rat plasma to which parathyroid extract (Lilly) was added either *in vitro* or *in vivo*. Similar experiments were carried out on fractions of human plasma with and without added parathyroid extract. Evidence is presented to suggest that endogenous rat plasma parathyroid hormone activity is normally associated with the α-globulin fraction. Cold ethanol fractionation of normal human plasma and of human plasma to which had been added parathyroid extract, resulted in localization of hormonal activity in the beta and gamma globulin fraction. The normal level of circulating parathyroid hormone activity in human plasma has been estimated to be of the order of 40 U.S.P. units per 100 ml.

IMPORTANT advances have been made in recent years in the understanding of the chemistry and physiological aspects of the parathyroid hormone. Very potent and highly purified preparations of the active principle have been obtained from glandular extracts (1, 2, 3, 4). New concepts and outlooks are being actively investigated to explain the mechanism of action of the parathyroid hormone on the molecular level (4, 5, 6).

Little information has yet been reported, however, on the detection of parathyroid hormone activity in the blood. A most recent study is that of Buckner and Nellor (7) who demonstrated the quantitative detection of parathyroid hormone-like activity in sera of rats with induced hyperparathyroidism.

The small amount of hormone present in the circulation, together with

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[2] Presented, in part, at the Meeting of the Federation of American Societies for Experimental Biology, April, 1960, Chicago.

[3] Public Health Service Research Fellow, National Heart Institute, 1958–1959.

[4] Present address, Department of Biochemistry, Division of Basic Health Sciences, Emory University, Atlanta, Georgia.

the relative insensitivity of biological assays has limited the number of techniques suitable for use in the study of plasma hormone characteristics. The one most commonly employed and applied with the greatest success for the detection, localization and estimation of various hormones in the plasma involves cold ethanol fractionation procedures under rigorously controlled conditions and the assaying of the resulting concentrated plasma fractions for hormonal activites. The present paper deals with the application of ammonium sulfate and cold ethanol fractionation procedures to the study of parathyroid hormone activities of human and rat plasma constituents.

EXPERIMENTAL

Biological Assay.—Test preparations were assessed for potency on the basis of the change in the serum calcium level of fasted, parathyroidectomized rats, six hours after intraperitoneal injection. Normal female rats of the Sprague-Dawley strain, weighing about 140 gm., were parathyroidectomized by cauterization and allowed to recover from the operation for a period of three days. Prior to use for assay, the animals were fasted for two nights and one day. The test dose did not exceed a volume of 5 ml. Initial studies showed that the maximum amount of plasma protein which could be administered intraperitoneally to these animals in a 5 ml. dose without visible toxic effects was 0.01 gram per gram of body weight. This dose limit was not exceeded in the biological assay of various preparations tested. Serum calcium was determined by the method of Natelson and Penniall (8). Protein was determined by the biuret method (9).

Preparation of Parathyroid Hormone-Enriched Rat Plasma. In order to study rat plasma parathyroid hormone activity, it was necessary to have available a rat plasma preparation possessing sufficient parathyroid activity to be detected by the biological assay system used. This was accomplished by administering several hundred U.S.P. units of commercial parathyroid extract (Injection Parathyroid, U.S.P., Lilly) intraperitoneally to adult female rats of the Sprague-Dawley strain weighing about 200 gm. each and exsanguinating these animals by cardiac puncture one to three hours later. When 4–5 ml. aliquots of plasma prepared in this manner were administered to assay animals, increases in serum calcium levels indicative of parathyroid activity were observed. In contrast, five ml. aliquots of plasma from untreated animals failed to elicit changes in serum calcium. The data are summarized in Table 1.

Ammonium Sulfate Fractionation of Parathyroid Hormone Enriched Rat Plasma. The fractionation was carried out using the method outlined by Koch and Hanke (10). The albumin and globulin fractions were dialyzed against distilled water in the cold until free from sulfate. Preliminary work established that such a dialysis procedure does not affect the potency of the parathyroid hormone-enriched rat plasma. After the dialysis, the protein fractions were lyophilized and stored in the Deepfreeze until used for assay. At that time, the globulin and albumin fractions were reconstituted to desired volumes with the use of 0.1% sodium chloride solution and water

TABLE 1. PARATHYROID HORMONE ACTIVITY OF PLASMA OF RATS INJECTED WITH PARATHYROID EXTRACT

No. of assay animals	Dose per animal	Change in serum calcium mg.%
8	5 ml.[a]	0.1 ± 0.1
5	4 ml.[b]	1.7 ± 0.8
6	5 ml.[c]	1.4 ± 0.4
6	5 ml.[d]	1.3 ± 0.5

[a] Freshly collected normal rat plasma.
[b] From plasma pool of 23 cc. collected one hour after injection of 400 U.S.P. units of parathyroid extract into each of nine rats.
[c] From plasma pool of 30 cc. collected one hour after injection of 500 U.S.P. units of parathyroid extract into each of eleven rats.
[d] From plasma pool of 36 cc. collected three hours after injection of 500 U.S.P. units of parathyroid extract into each of twelve rats.

respectively. Five ml aliquots were administered to each of a group of five animals in duplicate experiments. The results are summarized in Table 2.

Ammonium sulfate fractionation of hormonally active rat plasma resulted in a definite partition of hormonal activity between the albumin and globulin fractions. The parathyroid hormone activity is associated with the globulin fraction; no activity was detected in the albumin fraction.

Cold Ethanol Fractionation of Rat Plasma. The technique of cold ethanol fractionation was employed to study the distribution of parathyroid hormone activity in protein fractions of rat plasma. For this purpose, the method of Roberts and Kelley (11), a modification of the method of Ulrich, Li and Tarver (12) and of Cohn and his associates (13) was chosen, since it had been specifically developed for use in preparing rat plasma protein fractions. Two types of rat plasma were fractionated by this method: (1) rat plasma to which was added lyophilized parathyroid extract *in vitro* and (2) parathyroid hormone-enriched rat plasma prepared as previously described. In each case, plasma was collected, stored in the cold overnight and subjected to fractionation the following morning.

Preliminary work established that the storing of these preparations in this manner resulted in no detectable loss of biological activity. The following five fractions, with their major constituents, were obtained:

TABLE 2. PARATHYROID HORMONE ACTIVITY OF FRACTIONS OBTAINED BY AMMONIUM SULFATE FRACTIONATION OF PLASMA FROM RATS INJECTED WITH PARATHYROID EXTRACT*

Pool sample	Plasma fraction	Percent of total plasma protein	Dose per animal. mg. protein	No. of test animals	Change in serum calcium mg.%
1	Globulin	58.9	218	5	1.3 ± 0.5
	Albumin	38.9	336	5	−0.4 ± 0.6
2	Globulin	55.2	228	5	1.4 ± 0.2
	Albumin	35.7	352	5	−0.2 ± 0.2

* 50 ml. of pooled plasma from rats, each injected one hour previously with 500 U.S.P. units of parathyroid extract.

Fraction I—fibrinogen, Fraction II—alpha and beta globulins, Fraction SCA—beta globulins, Fraction IV—alpha globulins and Fraction V—albumins.

In the initial approach, 600 U.S.P. units of Parathyroid Extract were lyophilized and the residue was dissolved in 50 ml of rat plasma collected from female rats by cardiac puncture. Preparations obtained by the fractionation procedure of Roberts and Kelley (11) were collected in the refrigerated centrifuge at −5° C and then stored immediately in the freezer at −20° C. Prior to assay, each fraction was slowly warmed to room temperature and then reconstituted to a volume of 25 ml. with distilled water. Five ml. aliquots were administered to each animal of the five animal assay group. The experimental results are presented in Figure 1.

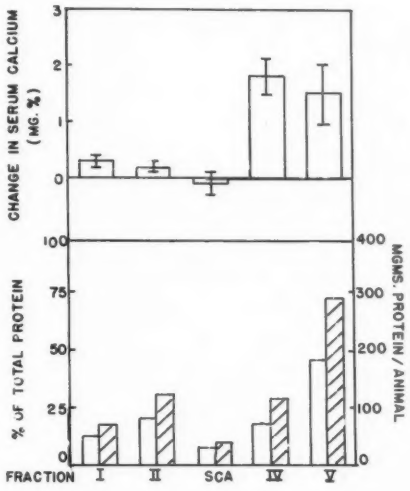

FIG. 1. Distribution of parathyroid hormone activity in plasma protein fractions obtained from 50 ml. of freshly collected rat plasma to which had been added parathyroid extract, 600 U.S.P. units *in vitro*. The open columns at the bottom of the figure represent the per cent of the total protein of the plasma contained in each fraction; the shaded columns indicate the amount of plasma administered per assay animal; the vertical lines in the upper figure represent the standard error of the mean.

The data indicate a definite partition of parathyroid hormone activity among the protein fractions of rat plasma, to which had been added parathyroid extract *in vitro* and subsequently subjected to cold ethanol fractionation. The parathyroid activity appears primarily associated with Fraction IV and Fraction V with no detectable activity being observed in Fractions I, II and SCA.

The administration of exogenous hormone to experimental animals and humans has been a technique utilized profitably by many workers to obtain a wide variety of information concerning plasma hormone characteristics. Accordingly, 500 U.S.P. units of parathyroid extract were administered intraperitoneally to individual female rats. After one hour, the animals were exsanguinated by cardiac puncture. The plasma was collected at the centrifuge and immediately placed in the freezer at −20° C

until fractionated. In each experiment performed, a sufficient number of animals was used to provide 50 ml. of plasma. The day following its collection, the hormonally active rat plasma was subjected to cold ethanol fractionation according to the method of Roberts and Kelley (11). The plasma protein fractions prepared in this manner were collected at the refrigerated centrifuge at $-5°$ C and immediately placed in the freezer unit until assayed. At that time the preparations were slowly warmed to room temperature and reconstituted to a total volume of 25 ml. with distilled water. Five ml. aliquots of the reconstituted fractions were administered intraperitoneally to each animal of a five-animal assay group (Fig. 2).

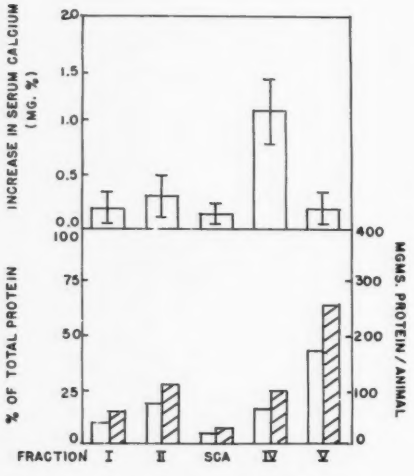

FIG. 2. Distribution of parathyroid hormone activity in plasma protein fractions obtained from hormonally active rat plasma. Identification of parameters same as in Fig. 1.

The data define a partition of parathyroid hormone activity among rat plasma protein fractions. This hormone activity appears to be localized primarily in Fraction IV which contains alpha-globulins. The distribution noted here is different from that observed when parathyroid extract was incubated with rat plasma *in vitro*, prior to fractionation.

Parathyroid Hormone Activity of Unfractionated Normal Human Plasma. Plasma obtained from a normal male donor at the Blood Bank, Cook County Hospital, Chicago, Illinois was lyophilized and tested for parathyroid hormone activity. When the protein present in 25 ml. of lyophilized plasma was administered to each of eight animals, it was found that the level of circulating parathyroid hormone was too small to elicit a response. The change of serum calcium amounted to -0.1 ± 0.2 mg. percent (mean $\pm$ standard error).

Cold Ethanol Fractionation Method of Cohn et al. (*Method 6*). Since it was not possible to detect plasma parathyroid hormone activity in lyophil-

ized human plasma, attention was focused on the application of the cold ethanol fractionation technique of Cohn *et al.* (13) designed specifically for fractionation of human plasma. By this technique, plasma is separated into five major fractions designated Fraction I—fibrinogen, Fraction II–III—beta and gamma globulins and beta-lipoproteins, Fraction IV-1—alpha globulins, Fraction IV-4—alpha globulins and Fraction V—albumins. The method was applied to normal human plasma and normal human plasma to which had been added parathyroid extract *in vitro*.

In the first experiments, 100 ml. of plasma were collected from a normal donor from the Cook County Hospital Blood Bank and in it were dissolved 500 units of lyophilized parathyroid extract. One hour after stirring the mixture was subjected to fractionation by Method 6 of Cohn and his associates (13). The fractions were collected at the refrigerated centrifuge and then placed in the freezer until assayed. The various fractions were reconstituted to a volume of 35 ml. with water and 5 ml. aliquots were administered to each of seven animals of an assay group. A composite picture of the results from two duplicate experiments is shown in Figure 3.

The experimental results indicate a localization of exogenous parathyroid hormone activity in Fraction II–III of normal human plasma, a fraction which is composed primarily of beta-globulins, gamma globulins and beta-lipoproteins. The biological activity recovered does not seem to be distributed to any significant extent among the other plasma protein fractions.

Cold Ethanol Fractionation of Normal Human Plasma. Concentrates of unfractionated normal human plasma were shown to lack sufficient parathyroid hormone potency to be detected by the assay system. It was now decided to apply cold ethanol fractionation techniques to normal plasma

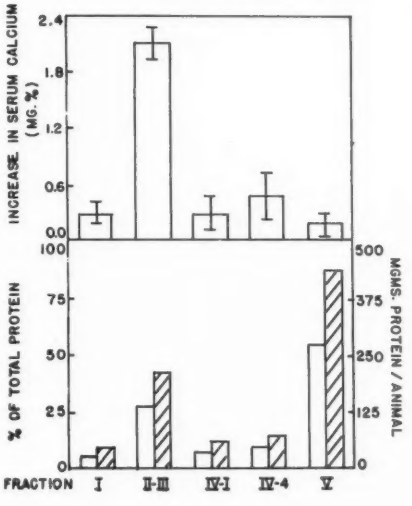

FIG. 3. Distribution of parathyroid hormone activity in plasma protein fractions obtained from normal human plasma (100 ml.) to which has been added parathyroid extract, *in vitro* (500 U.S.P. units). Identification of parameters, same as in Fig. 1.

and to assay the resulting protein fractions for parathyroid hormone activity.

For this experiment, 560 ml. of normal human plasma were obtained from two normal male donors at the Cook County Blood Bank. The plasma was then subjected to cold ethanol fractionation. The resulting fractions were lyophilized and stored in the freezer until used for assay at which time they were warmed to room temperature and reconstituted to sufficient volume so that each animal of the assay group received no more than 5 ml. aliquots of the reconstituted solution. This was done in all cases except that of Fraction V (albumins). Enough of this fraction was utilized for analysis so that each animal of a nine animal assay group received the maximum permissible dosage of protein. The biological assay data are given in Figure 4. The results indicate the detection of endogenous plasma

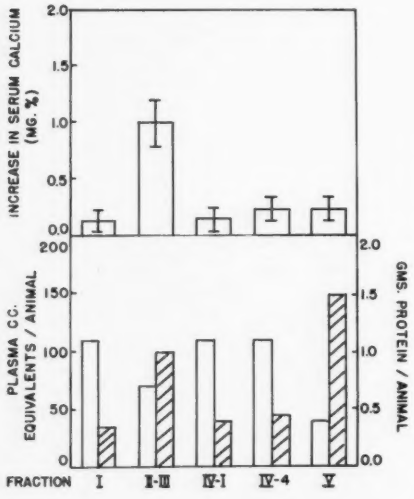

FIG. 4. Distribution of parathyroid hormone activity in plasma protein fractions obtained from normal human plasma. The open columns in the bottom figure represent the plasma cc. equivalents of each fraction administered per animal; the shaded columns indicate the grams of protein administered per test dose. The vertical lines in the upper figure represent the standard error of the mean.

parathyroid hormone activity in concentrates of protein in Fraction II–III of normal human plasma. No activity was detected in any other fractions assayed. These results were confirmed in a subsequent experiment in which 69 plasma cc. equivalents of Fraction II–III, comprising 25.4% of the total plasma protein, effected an increase in serum calcium of 1.0 ± 0.3 mg.% and 37 plasma cc. equivalents of Fractions I, IV-1, IV-4 and V, comprising 67.8% of the total plasma protein caused an insignificant increase of 0.2 ± 0.1 mg.% in the serum calcium level.

DISCUSSION

The intraperitoneal administration of parathyroid extract to rats resulted in a plasma containing detectable levels of parathyroid hormone

activity. Ammonium sulfate fractionation of this "hormonally active" rat plasma resulted in a localization of hormonal activity in the globulin precipitate. Cold ethanol fractionation of "hormonally active" rat plasma resulted in the localization of parathyroid hormone activity primarily in the alpha-globulin fraction. When parathyroid extract was incubated with freshly collected rat plasma, *in vitro,* hormonal activity was detected in the albumin fraction as well as in the alpha-globulin fraction. Accumulating evidence indicates that under normal conditions endogenous hormones may be associated, in transport, with specific plasma proteins, but when present in excessive quantities or introduced exogenously tend to "spill over" into associations with other plasma proteins (14). It is felt that such an interpretation may well apply to the findings reported here.

In the current study, parathyroid extract was also incubated with normal human plasma, *in vitro,* and the mixture was then subjected to cold ethanol fractionation. The distribution pattern observed indicated that the exogenous parathyroid hormone activity was localized in Fraction II–III, which consists mostly of beta and gamma globulins. When the cold ethanol fractionation procedure was applied to normal human plasma, the results obtained indicated the presence of endogenous plasma parathyroid hormone activity in Fraction II–III. It is interesting to note that Fraction II–III is the same fraction with which plasma ACTH activity (15) pituitary gonadotrophic activity (16), plasma thyrotrophic activity (17) and plasma insulin activity (18) were found to be associated following similar cold ethanol fractionation studies.

The mean response of the test group employed in the analysis of the potency of Fraction II–III is of the order which one would expect from about 25 U.S.P. units of parathyroid activity. Since this response was obtained from 70 plasma cc. equivalents of Fraction II–III protein, it may be estimated that normal human plasma contains of the order of 40 U.S.P. units of detectable endogenous parathyroid hormone activity per 100 ml. of plasma.

It should be pointed out that the parathyroid activity existing in the extracts employed is derived from beef glands and may not be identical to that produced in the rat or in man. Too, the parathyroid activity of the extract may represent a degradation product or products of the native hormone since acid extraction is employed in its production. In both instances, it might be expected that the active principles would bind to different proteins, than does the endogenous hormone. It can be noted, however, that in the case of the human plasma study, the distribution of activity among the plasma protein fractions isolated from the *in vitro* incubation of the parathyroid extract with plasma is the same as the distribution of the endogenous hormone-like activity.

Acknowledgments

The authors are indebted to Dr. John R. Tobin, Director, Cook County Blood Bank, Cook County Hospital, Chicago, Illinois for making available some of the human plasma utilized in this study. To Mr. J. E. Brackett and Dr. Glenn W. Irwin of Eli Lilly Company, Indianapolis, Indiana, indebtedness is also acknowledged for the generous supply of some of the Injection Parathyroid used in this work.

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QUANTITATIVE HISTOLOGICAL DISTRIBUTION OF CERTAIN PHOSPHORUS COMPOUNDS IN THE RAT ADRENAL IN VARIOUS FUNCTIONAL STATES[1,2]

LEONARD J. GREENBERG[3] AND DAVID GLICK

Histochemistry Laboratory, Department of Physiological Chemistry, The Medical School, University of Minnesota, Minneapolis, Minnesota

ABSTRACT

The quantitative histological distribution of inorganic and labile-ester phosphorus compounds in the adrenal gland of the male albino rat was studied. Subcutaneous injection of 25 mg. ACTH per kg. 3 hours prior to sacrifice lowered labile-ester phosphorus (predominantly ATP) in the fasciculata and elevated this fraction in the reticular-medullary border zone and medulla. Inorganic phosphorus was more uniformly distributed throughout the zones and the ACTH treatment produced some increase in all regions except outer fasciculata.

Analysis of whole-gland homogenates prepared from adrenals of untreated animals killed at noon and at midnight revealed a considerable decrease in labile-ester and a significant increase in inorganic phosphorus at midnight. Injection of ACTH largely abolished these day-night differences by decreasing the values of the former and increasing those of latter in the noon group. A series of subcutaneous cortisone injections (7 mg. cortisone acetate/kg. daily for 2 weeks) produced significant increases in concentration of the labile-esters in both groups, and in the inorganic fraction at noon. The treatment reduced the inorganic phosphorus of the whole gland at both periods. Six days after hypophysectomy little effect was detected either in the labile-ester or inorganic phosphorus concentration in adrenals from either the noon or midnight group, although a slight decrease in the ester content per gland at both periods was noted and variability in some of the data on the inorganic fraction was found. Comparison of labile-ester changes to those of coenzyme A (reported earlier) showed an inverse effect.

IN A previous study (1) the coenzyme A (CoA) concentration in the whole rat adrenal was found to be about 100% greater at midnight than at noon. Injection of ACTH abolished the day-night difference by producing maximal concentrations in both groups, and hypophysectomy also

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[3] Data in this paper were included in a thesis submitted in partial fulfillment of the requirements of the Graduate School of the University of Minnesota for the degree of Doctor of Philosophy.

minimized the difference. Subsequently, the circadian periodic change of CoA concentration was established by analyses at 4-hour intervals (2). Since phosphorus metabolism is intimately involved with energy producing reactions in various biological processes, including synthesis of CoA (3), the present investigation was initiated to elucidate physiological effects on phosphorus compounds in the adrenal zones, including relationships to the coenzyme. Quantitative histochemical distribution of inorganic and labile-ester phosphate in the adrenal gland of the rat in various functional states was studied. In addition, whole-gland homogenates were used to observe effects on phosphate concentrations of hypophysectomy and injections of ACTH and of cortisone in rats which were sacrificed at noon and midnight, periods of low and high bodily activity.

EXPERIMENTAL

Adrenal glands were obtained from male albino rats (Sprague-Dawley) weighing 300–400 gm., approximately 3 months old, purchased from Holtzman Co., Madison, Wis. Sham-operated and hypophysectomized rats were obtained from the Hormone Assay Laboratories, Inc., Chicago, Illinois. The animals were fed Purina fox chow and tap water *ad libitum*. They were housed in a constant climate room (78° ± 1° F, 30–50% relative humidity) with controlled illumination (lights on at 6:00 A.M., off at 6:00 P.M.) and instantaneous death was inflicted by a single hammer blow on the head at noon or midnight ±15 minutes. ACTH (25 mg./kg.) in 1 ml. of physiological saline solution was administered subcutaneously 3 hours prior to sacrifice of some of the animals. Cortisone acetate (7 mg./kg.) in saline suspension was administered subcutaneously everyday for 2 weeks prior to sacrifice.

Fresh frozen microtome sections (2.0 mm. diameter, 16 μ thick, 0.05 μl. vol.) were cut serially in a cryostat cabinet at −15° C and handled as described previously (4). One section was brushed flat on an albuminized glass slide and stained with toluidine blue for histological examination; the next 4 sections were placed in a reaction tube for analysis of phosphorus compounds and the protein precipitate obtained in the analysis was employed for determination of protein-nitrogen (P_N) by the bromsulfalein method (5). This sequence was followed through the entire cortex and into the medulla of the adrenal.

Phosphorus Determination

Inorganic and labile-ester phosphorus determination was based on the method of Sumner as modified by Taussky and Shorr (6), following a microadaptation developed in this laboratory by Dr. S. L. Bonting. In this method, phosphomolybdate is formed and reduced with ferrous sulfate to produce a blue color which is measured at 700 mμ.

Apparatus

Two types of reaction tubes were used: 4 mm. inner diameter, 27 mm. length, 132 μl. capacity; and 4 mm. inner diameter, 60 mm. length, 754 μl. capacity. Other micro-equipment was the same as that already described (7).

Reagents

1. 1% ammonium molybdate in 1.15N H_2SO_4.
2. 2N hydrochloric acid.
3. 5% trichloroacetic acid (TCA) solution.

4. Molybdate-ferrous sulfate mixture: Dissolve 0.4 gm. of ferrous sulfate ($FeSO_4 \cdot 7H_2O$) in 10 ml. of 1% ammonium molybdate solution. Prepare fresh immediately before each use.
5. Standard phosphate solutions: Dilute stock solution (1 mg./ml., 574 mg. $Na_2HPO_4 \cdot 2H_2O$/100 ml. water) with 5% TCA to give solutions of 2–8 μg/ml.

Procedure for Tissue Sections

1. Place 4 fresh-frozen tissue sections in 27 mm. reaction tubes.
2. Remove tubes from cryostat and add 100 μl. of cold 5% TCA to each tube; cap tubes and mix. (The supernantant fraction is designated as cold extractable phosphorus, CEP.)
3. Centrifuge at 2500×g. for 3 minutes.
4. Remove 50 μl. aliquots of the clear extracts and add each to a 27 mm. tube containing 50 μl. of molybdate-ferrous sulfate solution. Cap the tubes and mix. (Inasmuch as the color formed is stable from 2 minutes to 2 hours, these samples were not measured immediately.) Proceed with step 10.
5. Remove 25 μl. aliquots from the original cold TCA extract and add each to a 60 mm. reaction tube containing 25 μl. of 2N HCl. Cap and mix.
6. Place the tubes in a boiling water bath, allow 7 minutes for hydrolysis, and then transfer to an ice bath for 2 minutes. (This fraction is designated as hot extractable phosphorus, HEP. Extractable ester phosphorus, EEP, is calculated as HEP-CEP).
7. Evaporate to dryness in a vacuum desiccator over sodium hydroxide pellets and silica gel.
8. Add 50 μl. of 5% TCA to each tube; mix thoroughly.
9. Add 50 μl. of molybdate-ferrous sulfate solution to each tube; mix and cap.
10. Transfer the contents of each tube from steps 4 and 9 to a microcuvette and determine absorbancy at 700 mμ.
11. Run a reagent blank by measuring the absorbancy of a mixture of 50 μl. 5% TCA and 50 μl. molybdate-ferrous sulfate solution.
12. Determine P content from a calibration graph constructed for each experiment using 3 standard solutions.
13. Evaporate to dryness (as in step 7) the contents remaining in the original tubes with the tissue sections and determine P_N on the residue.

Procedure for Homogenates

Homogenize single adrenal glands in cold 5% TCA for 1 minute using the procedure employed in the CoA study (1). Centrifuge at 2500×g. for 3 minutes and carry out analysis as described for tissue sections.

RESULTS AND DISCUSSION

From Figure 1 it is apparent that CEP is rather uniformly distributed in all zones. There is, however, a distinct peak in HEP, and consequently in EEP, in the fascicular zone. Furthermore, ACTH administration results in a depression of this peak and an elevation of both HEP and EEP in the reticular-medullary border zone and medulla, while a small increase in CEP occurs in all zones except outer fasciculata. There might be a connection between these data and the possibility presented by Symington (8) that precursors in the fasciculata are utilized for hormone synthesis in the reticularis when the adrenal is stimulated by ACTH or stress conditions.

Splanchnic nerve stimulation in cats by morphine and in rats by insulin

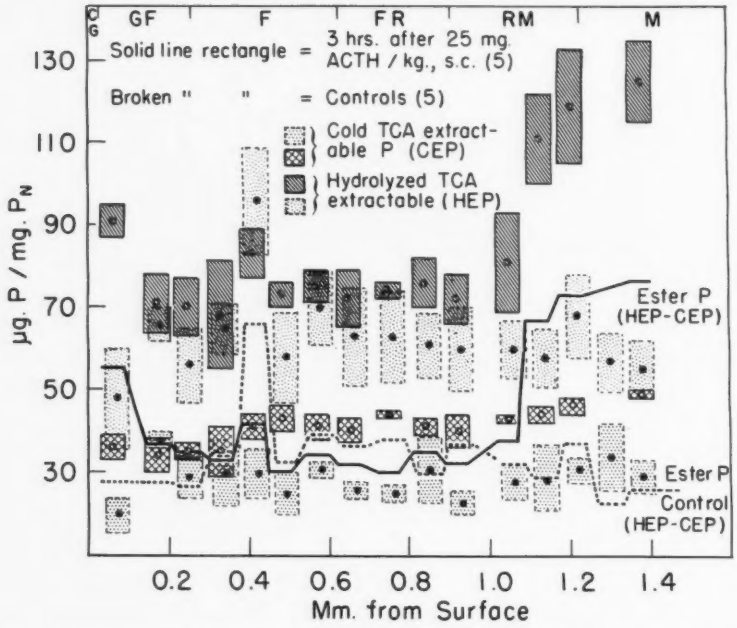

FIG. 1. Quantitative histological distribution of inorganic and labile-ester phosphorus in the adrenal gland of male albino rats and the effect of ACTH treatment. Four microtome sections (each 2 mm. diameter, 16 μ thick, 0.05 μl. volume) were used for each analysis of phosphorus compounds and protein-nitrogen. The height of each rectangle represents twice the standard error of the mean value, the width represents the thickness of 4 sections, the numbers in parenthesis indicate the number of glands used to obtain the composite data. Regions marked C, G, F, R and M denote respectively, capsule, glomerulosa, fasciculata, reticularis and medulla. Mixed zones are designated by both letters.

TABLE 1. EFFECT OF TREATMENT AND TIME OF SACRIFICE ON MEAN CONCENTRATIONS OF INORGANIC PHOSPHORUS IN WHOLE RIGHT ADRENAL GLANDS OF MALE ALBINO RATS

Treatment	No. adrenals	μg CEP / gland	±S.E.	p*	μg CEP / mg. wet wt.	±S.E.	p*	μg CEP / mg. P_N	±S.E.	p*
Sacrificed at Noon										
Untreated	5	9.7	0.38		0.39	0.006		19	0.6	
ACTH†	5	22.1	3.13	0.004	0.86	0.009	<0.001	22	0.4	0.004
Cortisone‡	6	6.6	0.39	<0.001	0.47	0.015	<0.001	33	2.0	<0.001
Sham Hypophysectomy	4	13.5	1.80		0.58	0.073		23	2.0	
Hypophysectomy	4	11.7	1.65	0.5	0.82	0.033	0.2	38	3.0	0.006
Sacrificed at Midnight										
Untreated	5	15.7	0.89		0.67	0.037		29	2.6	
ACTH†	6	17.5	0.71	0.2	0.68	0.013	>0.6	31	1.7	>0.6
Cortisone‡	4	5.3	0.70	<0.001	0.59	0.052	>0.6	28	5.2	>0.6
Sham Hypophysectomy	4	11.3	0.86		0.57	0.035		24	3.2	
Hypophysectomy	4	6.9	0.96	0.01	0.58	0.028	>0.6	26	4.5	>0.6

* p values referred to corresponding data on untreated rats; hypophysectomy data referred to that on sham-operated rats, animals sacrificed 6 days after operation.
† Sacrificed 3 hours after subcutaneous injection of 25 mg. ACTH/kg.
‡ Sacrificed after 2 weeks of daily subcutaneous injections of 7 mg. cortisone acetate/kg.

(9), and in sheep by insulin (10), was reported to effect a decrease in ATP concentration in the adrenal medulla with a corresponding fall in catechol amine concentration. The relationship of these results to the increase in medullary EEP following ACTH treatment observed in rats in the present investigation requires further study.

The results of the investigation on CEP and EEP in whole adrenals are summarized in Tables 1 and 2, respectively. Significant increases in CEP values in adrenals from untreated rats at midnight compared to noon, and at noon following ACTH administration, were found. Corresponding significant decreases were observed in EEP values. Differences between control values and those following ACTH injection for either CEP or EEP were not significant at midnight.

TABLE 2. EFFECT OF TREATMENT AND TIME OF SACRIFICE ON MEAN CONCENTRATIONS OF LABILE-ESTER PHOSPHORUS IN WHOLE RIGHT ADRENAL GLANDS OF MALE ALBINO RATS

Treatment	No. adrenals	μg EEP / gland	±S.E.	p*	μg EEP / mg. wet wt.	±S.E.	p*	μg EEP / mg. P_N	±S.E.	p*
Sacrificed at Noon										
Untreated	5	7.8	0.51		0.31	0.014		15	1.1	
ACTH†	5	2.7	0.34	0.001	0.11	0.016	<0.001	3	0.6	<0.001
Cortisone‡	6	6.2	0.50	>0.14	0.57	0.026	<0.001	30	1.0	0.004
Sham Hypophysectomy	4	1.3	0.24		0.06	0.010		2	0.4	
Hypophysectomy	4	0.8	0.15	0.5	0.06	0.009	>0.6	3	0.4	>0.6
Sacrificed at Midnight										
Untreated	5	3.3	0.74		0.14	0.032		6	0.5	
ACTH†	6	3.7	0.60	>0.6	0.15	0.026	>0.6	6	1.1	>0.6
Cortisone‡	4	7.9	0.70	0.006	0.40	0.057	0.013	42	3.1	0.001
Sham Hypophysectomy	4	2.1	0.20		0.11	0.017		4	0.7	
Hypophysectomy	4	1.6	0.24	0.5	0.14	0.021	0.3	6	1.2	0.2

* p values referred to corresponding data on untreated rats; hypophysectomy data referred to that on sham-operated rats, animals sacrificed 6 days after operation.
† Sacrificed 3 hours after subcutaneous injection of 25 mg. ACTH/kg.
‡ Sacrificed after 2 weeks of daily subcutaneous injections of 7 mg. cortisone acetate/kg.

Cortisone treatment resulted in a significant decrease in whole gland CEP values both at noon and midnight, while an increase in the EEP value occured only at midnight. Little cortisone effect was found on the CEP concentrations at midnight although significant increases were observed at noon. On the other hand, increases in EEP were seen in both noon and midnight concentrations. This complex relationship between the CEP and EEP values is not understood at present.

Reiss and Halkerston (11) found that there was an increase in incorporation of inorganic P^{32} into an acid-soluble fraction of adrenals from rats exposed to cold. This was confirmed by Nicholls and Rossiter (12) who also showed increased incorporation of P^{32} into the separate inorganic and labile-ester phosphorus fractions. Injection of ACTH was shown to cause an effect similar to that of exposure to cold (13–15). Nicholls *et al.* (16) concluded that the changes in adrenal phosphorus metabolism, mediated by endogenous ACTH, were an increase in the rate at which inorganic P^{32} passes from the extracellular to the intracellular space and a net synthesis

of organic-phosphorus compounds. Although differences in experimental conditions in the present study do not justify direct comparison to data from the experiments on P^{32} incorporation, both sets of data indicate that ACTH influences, and perhaps regulates, inorganic and labile-ester phosphate concentrations in the rodent adrenal.

It is also of interest that conditions, such as day-night differences and ACTH stimulation, produce opposite effects on EEP concentrations and CoA, although these changes are not related stoichiometrically and ATP is required for many reactions in addition to those involving sythesis of CoA.

With the exception of an increase in the CEP per unit P_N at noon, and a tendency to a decrease in total gland CEP at midnight, no significant changes in either CEP or EEP were apparent between the sham-operated and hypophysectomy groups, perhaps because stress effects were operative in both. Hypophysectomy was reported to result in decreased incorporation of P^{32} into inorganic phosphate (13, 14), total acid-soluble phosphorus compounds (11), phospholipid (17), and RNA (18). Subsequent injection of ACTH reversed these changes in all cases. In the experiments reported here, those on adrenals from the sham-operated and hypophysectomized rats can not properly be compared with the others as the aminals were of different stocks.

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THE ISOLATION OF ARGININE VASOTOCIN FROM FISH PITUITARY GLANDS

HOWARD RASMUSSEN[1] AND LYMAN CRAIG
Rockefeller Institute, New York, N.Y.

ABSTRACT

A neurohypophyseal hormone was isolated from Hake pituitary powder by means of countercurrent distribution and column chromatography. This hormone has the biological potency and amino acid composition of arginine vasotocin.

FOLLOWING their classic work on the isolation, characterization and synthesis of oxytocin and vasopressin (1), du Vigneaud and his collaborators undertook the synthesis of a variety of analogs of these octapeptides (2). One of the first to be prepared was arginine vasotocin (3). This interesting compound represents the side chain of arginine vasopressin and the ring of oxytocin. Studies carried out by Munsick, Sawyer and van Dyke revealed that this synthetic agent had marked physiologic activity in several tissues (4). From comparative pharmacologic studies, Sawyer was led to the conslusion that arginine vasotocin was the natural neurohypophyseal hormone of many nonmammalian verterbrates (5). This conclusion has recently been directly confirmed by Acher *et al.* who have isolated and tentatively identified arginine vasotocin from the pituitaries of frogs (*Rana esculenta*) (6) and chickens (7), and by Heller and Pickering (8) who have tentatively isolated this hormone from Pollack pituitaries. The purpose of this report is to describe the isolation of this hormone from the pituitary glands of Hake, *Merluccius Vulgaris.*

MATERIAL AND METHODS

Hormonal assays were carried out on the isolated urinary bladder of the toad (*Bufo Marimus*). Toads weighing 200–230 gm. were kept hydrated but not fed. Immediately before use they were pithed and the bladder removed in two halves. The bladders were mounted in a bath according to the previously described modification (9) of the technique of Bentley (10). The weight loss (mg./min.) of the mounted bladder was recorded every 20 minutes. During the two control periods the usual weight loss was 1.5 mg./min.: following a maximal hormonal stimulation the weight loss averaged 30–35 mg./min. Between these extremes, the weight loss was proportional to the log dose of hormone.

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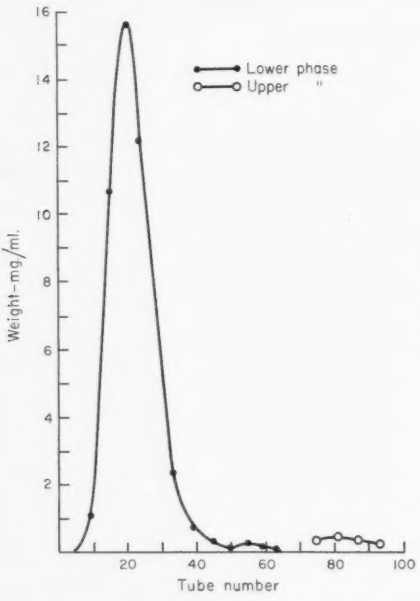

FIG. 1. The countercurrent distribution of 538 mg. of a crude Hake pituitary powder in a system of 0.15 M ammonium acetate in 0.1% acetic acid: n-butanol: pyridine (12.5:5.4).

The log dose response of the unknown was compared to that of arginine vasotocin, arginine vasopressin, and oxytocin.

The crude fish pituitary powders were obtained from Professor A. E. Wilhelmi of the Emory University. They were fractions prepared during the course of his work on the isolation of the growth principle from these glands. Many of the fractions surveyed had moderate hormonal activity but only one had appreciable activity. This had been prepared by overnight extraction of freshly ground pituitaries with 0.25 M ammonium sulfate; precipitation of the material with 1.25 M ammonium sulfate; resolution in water at pH4; reprecipitation with 1.25 M ammoniumsulfate; followed by dialysis and lyophilization. The resulting powder was the starting material for the present work.

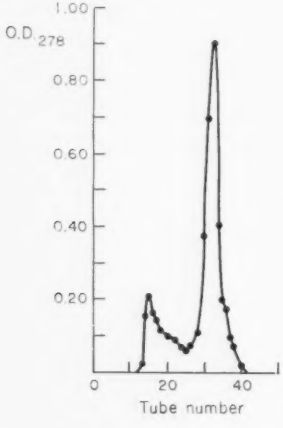

FIG. 2. Chromatography of fractions 48–63 (Fig. 1) on a 0.9×120 cm. column of Sephadex (G-25).

Countercurrent distribution was carried out by methods previously described (11). The solvent system employed was composed of n-butanol: 0.15 M ammonium acetate in 0.1% acetic acid: pyridine (5:12·5:4). Analysis for polypeptides and proteins was done either by weight (12) or ultraviolet absorption (278 mμ) using a Beckman D.U. spectrophotometer. Chromatography was performed on a 0.9×120 cm. column of Sephadex (G-25)[2] using 0.2 M acetic acid as the eluent. The Sephadex was screened dry through a 200 mesh sieve. The material passing through the sieve was taken up in 0.2 M acetic acid, was allowed to settle, and the fines removed by decantation. This procedure was repeated several times. The resin was poured into the column in 30 cm. sections. The eluent was allowed to flow without pressure, attaining a rate of 2–3 ml./hr. The samples were applied to the top of the column in 1.5–2.0 ml. of 0.2 M acetic acid. A sample of the final product was hydrolyzed in 6.6N HCl in a sealed evacuated tube at 110° for 70 hours. Amino acid analysis was carried out by the method of Spackman, Stein and Moore (13).

RESULTS

When 538 mg. of crude fish powder were distributed for 94 transfers, the pattern shown in Figure 1 was observed. Only in tubes 48 to 63 was significant hormonal activity detected. This material represents 1–2% of the weight and 80–85% of the estimated hormonal activity. It was recovered, the organic solvents removed by evaporation and the solution lyophilized. The lyophilized powder was taken up in 1.5 ml. of 0.2 M acetic acid and

[2] Manufactured by Pharmacia, Uppsala, Sweden.

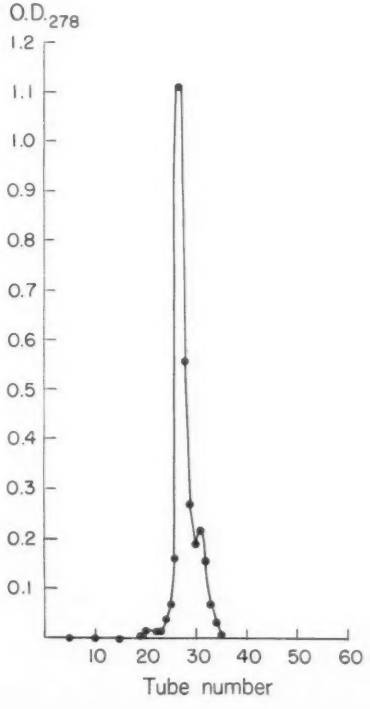

FIG. 3. Rechromatography of fractions 28–36 on Sephadex.

applied to a 0.9×120 cm. column of Sephadex (G-25). The effluent pattern obtained is shown in Figure 2. The material in tubes 28–36 was recovered, lyophilized and rechromatographed as shown in Figure 3. The material (3.5 mg.) in tubes 26–29 was recovered. It had a biologic potency of 95 ±20% of a synthetic sample of arginine vasotocin; a potency approximately 20 times greater than oxytocin; and 75 times greater than arginine vasopressin. These relative potencies are comparable to those found with the synthetic vasotocin.

The results of the amino acid analysis are recorded in Table 1. The isolated material has a composition similar to that of arginine vasotocin, although the yields of tyrosine and cystine were somewhat low and that of ammonia high. In addition a small amount of serine was detected. No other amino acids were found.

TABLE 1. THE AMINO ACID COMPOSITION OF MATERIAL FROM FRACTIONS 26–29, FIG. 3

The recovered amino acids and ammonia accounted for 99% of the nitrogen and 94% of the weight of the sample.

Amino acid	Residues per molecule	
	Unknown	Arginine Vasotocin
Ammonia	3.80 (3)	3
Arginine	0.82 (1)	1
Aspartic	1.00 (1)	1
Serine	0.19	0
Glutamic	0.89 (1)	1
Proline	1.15 (1)	1
½ Cystine	1.65 (2)	2
Isoleucine	1.12 (1)	1
Tyrosine	0.77 (1)	1

DISCUSSION

Because of the scarcity of material, it was not possible to characterize completely the chromatographically purified hormone. However, the biological data and the results of amino acid analysis indicate that the pituitaries of these fish contain arginine vasotocin. The low yields of cystine and tyrosine are undoubtedly due to oxidative loss of these amino acids during acid hydrolysis. Also, the serine probably arose from the cystine during acid hydrolysis. A similar phenomenon has been observed by du Vigneaud (14) during the hydrolysis of neurohypophyseal peptides, and we have noted it during the hydrolysis of a sample of pure oxytocin.

On the basis of Professor Wilhelmi's calculations, it can be estimated that these Hake pituitaries contain at least 30 mg. of arginine vasotocin per kilogram of glands. Whether other neurohypophyseal analogs are present in these glands can not be decided on the basis of our evidence.

Our data confirm the supposition of Sawyer that fish pituitaries contain arginine vasotocin (5). They indicate, as do the data of Acher *et al.* (6, 7) and that of Heller and Pickering (8), that this hormone is quite widespread in the nonmammalian vertebrates.

Acknowledgment

We are grateful to Professor Vincent du Vigneaud for his interest and his gift of the synthetic arginine vasotocin used in this work. We are particularly grateful to Professor A. E. Wilhelmi for his generous supply of crude pituitary fractions.

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NOTES AND COMMENTS

EFFECT OF ADMINISTRATION OF HUMAN SERUM THYROXINE-BINDING GLOBULIN ON THE DISAPPEARANCE RATES OF THYROID HORMONES IN THE CHICKEN[1]

ABSTRACT

The disappearance rates of intracardiac administered I^{131}-labeled L-thyroxine (T-4) and 3,5,3′-triiodo-L-thyronine (T-3) were measured by the whole body counting technique in young White Leghorn cockerels. The disappearance rate was expressed as a half-time for both a fast component (0–24) and a slow componet (24–54 hours). The half-times for both components were not different for the two hormones. When small amounts of a preparation of enriched human serum thyroxine-binding globulin (TBG) was mixed with radiothyroxine before administration of the labeled hormones, the half-time of the labeled T-4 in the first 24 hours was increased from 9.4 ± 0.5 to 14.2 ± 1.8 hours. TBG did not affect the disappearance rate of labeled triiodothyronine when the two were similarly injected nor did TBG affect the half-time of the slow componet of T-4 or T-3. Small amounts of TBG failed to influence the half-times of either of the two hormones if the TBG were administered 15 minutes or 24 hours after administration of the hormones, suggesting either that the hormone or the human serum protein was removed from the circulation before any combination of hormone and human serum protein could occur. The selective increase in half-time of T-4 by human TBG to a higher value than the half-time of T-3 in birds complements an earlier observation where the similar half-times of the two hormones in birds was attributed to a demonstrated absence of TBG in bird serum. The results also support the view that the relative potencies of the two hormones in different species may be in part, a reflection of rates of their distribution to the tissues.

Unlike the 5 to 8-fold higher activity in mammals (1), 3,5,3′-triiodo-L-thyronine (T-3) exhibits the same biological potency or a potency no greater than L-thyroxine (T-4) in a variety of tests in the chicken (2, 3). This difference in relative potencies of the two thyroid hormones was explained on the basis of a fundamental difference in the pattern of transport of the circulating thyroid hormones in the two species (4). Chicken and duck sera did not contain the alpha-globulin-type thyroxine-binding globulin (TBG) commonly found in the blood of most vertebrate species (1, 5, 6). In mammalian serum, TBG, which carries the major part of circulating thyroid hormone, has a 4-fold higher affinity for T-4 than T-3, whereas in avian sera the two hormones are bound almost equally as firmly. Consequently, thyroxine and triiodothyronine exhibited identical disappearance rates in the chicken, whereas in most mammals T-3 disappears at a 2–3 times faster rate than T-4. It was suggested therefore that the relative ease with which the two thyroid hormones diffuse into tissues was correlated with their relative biological potencies (4).

The validity of the above explanation would be enhanced if it were possible to modify the metabolism of thyroid hormones in birds by the administration of mammalian serum TBG. Recently, one of us has succeeded in purifying human serum TBG (7). The aim of this communication is to describe the effect of administration of an enriched TBG preparation on the disappearance rates of thyroxine and triiodothyronine in the chicken.

EXPERIMENTAL

Human serum thyroxine-binding globulin was purified by a method to be described elsewhere. Although the preparations of TBG used in this work were not homogeneous, they were free from the other major thyroxine-binding proteins in human serum, namely, albumin

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TABLE 1. EFFECT OF SIMULTANEOUS ADMINISTRATION OF A HUMAN SERUM TBG PREPARATION ON THE DISAPPEARANCE RATES OF I^{131}-LABELED L-THYROXINE (T-4) AND 3,5,3′-TRIIODO-L-THYRONINE (T-3) IN THE CHICKEN

Substance	Amount injected μg	TBG prep. mixed mg.	Disappearance rate or half-time (hours) Mean ±S.D.	
			0–24 hours	24–54 hours
T-4	0.077	None	9.4 ±0.5 (11)	24 ±5 (6)
T-4	0.060	5.0	14.2 ±1.8 (5)	25 ±8 (5)
T-3	0.233	None	8.6 ±1.4 (11)	27 ±8 (6)
T-3	0.070	5.0	8.6 ±0.5 (5)	18 ±3 (5)

When mean is underlined once, different from value to the left, when doubly underlined, different from value above, by t-test, P = 0.01.
Numbers in parentheses ( ) refer to the sample size.

and "thyroxine-binding prealbumin" (TBPA) (5, 8). Only 10–20 percent of total protein in preparations used here corresponded to an active thyroxine-binding component whose nature has been described (9).

Two types of experiments were performed. In the first, human TBG was mixed with radioactive thyroxine and triiodothyronine before administration to the birds. In the second type, TBG was administered separately by the intravenous route in 0.10 cc. of saline and after the labeled hormones. The amount of either hormone or TBG is given in Table 1 and 2. Groups of White Leghorn cockerels, average weight of 125 gm., were injected in the heart with 0.05 ml. of 0.15 M NaCl containing tracer amounts of I^{131}-labeled L-thyroxine[2] or 3,5,3′-triiodo-L-thyronine.[2] Each bird was injected subcutaneously with 2ml. of a 0.025% solution of propylthiouracil at 1 hour before the administration of the labeled hormones, and then at 4–12 intervals. The I^{131} retained by each bird was measured with a whole body well-type gamma counter against suitable standards, beginning 15 minutes after administration of the hormone and then at convenient hourly intervals over the next 54 hours until at least 12 radioactivity measurements were made on each bird. Using the first count as 100% and the succeeding counts as a percentage of this, the disappearance rates of the two hormones were expressed as half-time for the first 24 hours (fast component) and for 25–54 hours (slow component). The ultimate meaning of the 2 componet curve is not completely understood but it is a common finding (1) and may represent in the faster component the passage of hormone from the blood into tissues and organs and in the slower component the metabolism of the hormone by the cells.

TABLE 2. LACK OF EFFECT OF HUMAN TBG ON THE DISAPPEARANCE RATES OF L-THYROXINE (T-4) AND 3,5,3′-TRIIODO-L-THYRONINE (T-3) IN THE CHICKEN, WHEN ADMINISTERED 15 MINUTES AND 24 HOURS AFTER THE HORMONES

Substance	Amount injected μg	TBG injected mg.	Time after hormone injection	Disappearance rate or half-time (hours) Mean ±S.D.	
				0–24 hours	24–54 hours
T-4	0.037	None	—	11.0 ±2.1 (2)	16 ±3 (2)
T-4	0.037	8.0	15 min.	10.3 ±3.4 (6)	17 ±1 (6)
T-4	0.077	None	—	9.4 ±0.5 (11)	24 ±5 (6)
T-4	0.077	5.0	24 hrs.	—	17 ±3 (5)
T-3	0.034	None	—	9.5 ±1.5 (2)	19 ±3 (2)
T-3	0.034	8.0	15 min.	8.0 ±1.7 (2)	17 ±1 (2)
T-3	0.233	None	—	8.6 ±1.4 (11)	27 ±8 (6)
T-3	0.233	5.0	24 hrs.	—	18 ±3 (5)

[2] Purchased from Abbott Laboratories, Oak Ridge, Tenn. Specific radioactivity: L-thyroxine, 46–54 μc/μg; 3,5,3′-triiodo-L-thyronine, 25–33 μc/μg. Both substances were checked chromatographically before use and found not to contain more than 7% of the radioactivity as iodide.

RESULTS AND DISCUSSION

Table 1 summarizes the results obtained in birds to whom a previously mixed preparation of TBG and either T-4 or T-3 was administered, or the hormones were given without the TBG. As with previous observations (4) the disappearance rates of the two hormones are not significantly different in the absence of the human TBG preparation. However the half-time of the faster component of thyroxine was markedly increased if the hormone was administered after mixing it *in vitro* with a small amount of TBG. The human serum protein did not influence the disappearance rate of triiodothyronine. The relative difference in the half-times of the two hormones for the first 24 hours in the chicken caused by the presence of human TBG resembles the pattern observed in mammalian species (1, 6).

In the above experiments, the administration of TBG failed to alter the disappearance rates of both the hormones for the slower component of 24–54 hours. This suggested that human TBG was very rapidly removed from circulation in the chicken. Experiments in which TBG was administered after the labeled hormones supported this explanation (Table 2). If TBG was injected even only 15 mins. after T-4 or T-3 it failed to influence significantly the rate at which the hormones were metabolized. Considering the small amount of human serum protein administered and that only 10–15% of it represented TBG, it is likely that the amount of human TBG circulating in chicken blood was too small to affect the distribution of the hormones when measured over a relatively long period of time. It is possible also that during the interval between hormone administration and TBG administration relatively large amounts of the hormone had left the blood and thus was not accessible to TBG. When larger amounts of human TBG are available, it will be important to study its effect at a sustatined high blood level in the chicken.

The selective retardation of the metabolism of thyroxine in the first series of experiments complements previous observations on the absence in chicken blood of an alpha-globulin-type TBG present in mammals which binds thyroxine more firmly than triiodothyronine. In the previous study (4), there was a relative absence of TBG in chicken blood and the half-times of T-4 and T-3 were similar while in the present study when TBG was administered, the half-time of T-4 was increased. The absence of TBG in duck serum has recently been confirmed (10). The apparent relation of half-time of the 2 thyroid hormones and their biological activity, similar half-times and biological activities in birds while in mammals a slower half-time and lower biological activity of T-4 than of T-3, support the view that the pattern of their distribution to tissues rather than specific differences in reacting with intracellular enzymes is the explanation, at least in part, for the difference observed in the biological potencies of thyroid hormones (6, 11).

C. J. SHELLABARGER[3] AND J. R. TATA[4]

Medical Department
Brookhaven National Laboratory
Upton, L. I., New York
and
National Institute for Medical Research
Mill Hill, London N. W. 7, England

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[3] Present address: Kresge Radioisotope Laboratories, University of Michigan Medical School, Ann Arbor, Michigan.

[4] Beit Memorial Fellow.

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INTERACTION OF RELAXIN AND OVARIAN STEROID HORMONES OF UTERUS OF RAT[1]

ABSTRACT

Intact and ovariectomized rats (with or without 10 days involution period) were given estradiol benzoate (EB) alone (0.2 μg. or 1 μg daily) and concurrent with 15 and 45 GPU of relaxin (R) for periods of about 10 days. In intact animals EB stimulated a slight increase in both wet and dry uterine weight but the combination of EB and R stimulated significant increases both in comparison with control or EB-treated animals. Relaxin augmented the uterine growth effect of estrogen and induced a hydration in the uterus. Ovariectomized animals showed a marked uterine involution which was restored with EB, but combination of EB and R failed to stimulate uterine growth comparable to that in intact animals during the period of injection, due, it is believed, to lack of endogenous progesterone. Progesterone alone had no appreciable effect on uterine growth, and rather reduced effect of estrogen. However, it enhanced action of combination of estrogen and R in uterine growth indicating these three hormones are required for complete growth of uterus.

In the past, the principal function assigned to relaxin has been the relaxative effect on the pubic symphysis associated with alteration in the collagen and water content of the pubic ligament (1). More recently, the role of relaxin in the physiology of the uterus and mammary gland has been studied. It has been shown that relaxin, alone or in synergism with estrogen, influences uterine growth, water and glycogen content, contractility and cervical dilation (2, 3, 4, 5, 6, 7, 8). Evidence of synergism between progesterone and relaxin in modifying weight and composition of uterus of the estrogen-primed, spayed rat was also presented (6). Mammary gland lobule-alveolar growth has been reported (9, 10). These effects indicate that relaxin may play important roles during pregnancy as well as to facilitate parturition. Present study is concerned with interaction of relaxin (R), estradiol benzoate (EB) and progesterone (P) on growth of the uterus of normal and ovariectomized rats. The study was, in part, carried out concurrently with effect of R on mammary gland growth in the rat (11).

MATERIALS AND METHODS

Adult female rats of the Sprague-Dawley-Rolfsmeyer strain weighing approximately 230 to 250 gm., kept in a room artificially illuminated during daylight hours, at a uniform

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temperature of 78 ± 1° F, were fed commercial rat feed. First groups were ovariectomized 2 weeks previous to start of experiment, whereas second groups were started immediately. Estradiol benzoate (EB) was injected daily at rate of 1 μg for 7 days to intact groups and for 9 days to ovariectomized groups concomitant with 30 and 90 GPU of relaxin suspended in 0.1 ml. sesame oil plus 5% beeswax carrier every other day.[2] Progesterone (P) alone and in combination was administered also. To study influence of ovariectomy on uterine involution, virgin albino rats weighing 120 to 140 gm. were used. One lot of intact animals served as controls. Hormone treatment was the same as in the previous experiment. On the day following final injection, the animals were sacrificed and the uterus was dissected free of mesentery, incised longitudinally and blotted to absorb fluid, weighed, dried in an oven at 100° C for 2 days and reweighed.

RESULTS

Injection of 1 μg estradiol benzoate in intact rats stimulated a slight increase in both wet and dry weight of uterus. This was further increased significantly by concurrent administration of EB and R, in comparison with control and EB injected group. No difference between groups receiving doses of R with EB was observed (Table 1).

After 2 weeks, ovariectomized control animals showed marked uterine involution on basis of wet and dry weight in intact control group. Injection of EB restored both wet and dry weight comparable to that of the intact control group and increased significantly these weights in comparison with ovariectomized control group ($P = 0.001$). Increase in

TABLE 1. EFFECT OF RELAXIN ON UTERINE GROWTH AND WATER CONTENT IN RAT
(Involution 2 weeks)

Group	Treatment Amt. × Days	No. of rats	Uterus		
			Wet wt. (mg.) Mean ± S.E.	Dry wt. (mg.) Mean ± S.E.	Water content (%) Mean ± S.E.
Intact Control		10	486 ± 39	83.8 ± 5.6	82.3 ± .5
	EB 1 μg ×7	10	511 ± 35	95.2 ± 5.2	81.2 ± .3
	EB 1 μg R 15 GPU ×7	10	685 ± 33[2,5]	114.3 ± 5.0[3,4]	83.2 ± .2[6]
	EB 1 μg R 45 GPU ×7	10	679 ± 32[2,5]	107.6 ± 4.4[2]	84.0 ± .3[1,6]
Ovariectomized Control		10	134 ± 11	25.2 ± 2.6	81.3 ± .6
	EB 1 μg ×9	10	456 ± 16[3]	82.6 ± 6.4[3]	81.9 ± .3
	EB 1 μg R 15 GPU ×9	11	491 ± 24[3]	88.0 ± 4.9[3]	82.0 ± .4
	EB 1 μg R 45 GPU ×9	10	500 ± 31[3]	85.9 ± 6.1[3]	82.8 ± .4

EB = Estradiol Benzoate.
R = Relaxin.
[1] Significant at 5% to control group.
[2] Significant at 1% to control group.
[3] Significant at 0.1% to control group.
[4] Significant at 2% to estrogen group.
[5] Significant at 1% to estrogen group.
[6] Significant at 0.1% to estrogen group.

[2] Relaxin preparation kindly supplied by Dr. R. L. Kroc, Chilcott Lab., Morris Plains, N. J., assayed 30 GPU/mg.

wet and dry uterine weight under influence of concurrent EB and R injections in the ovariectomized groups was less significant than in intact group. Comparing corresponding groups of intact and castrate, significant differences in wet and dry weight were found in both groups with combination of EB and R.

Either EB or combination of EB and R had a tendency to increase water content of uterus in castrate groups, however, only in intact group injected with EB and R was the water content of the uterus increased significantly in comparison to control group or EB injected group.

When ovarian hormones were injected immediately following ovariectomy neither R nor P had appreciable effect on uterine growth without EB. Estrogen stimulated increased wet and dry uterine weight. Relaxin augmented effect of EB on wet uterine

TABLE 2. UTERINE RESPONSE OF RATS TO OVARIAN HORMONES
(Injected for 9 days after ovariectomy)

Treatment	Group no.	No. of animals	Uterus		
			Wet wt. (mg.) Mean ±S.E.	Dry wt. (mg.) Mean ±S.E.	Water content (%) Mean ±S.E.
Intact Control	1	8	157.8±18.8	34.1±5.3	78.2±1.1
R:15 GPU/day	2	5	221.4±55.9	40.8±9.9	81.4± .2
EB:1 μg/day+R:15 GPU/day	3	8	449.3±41.6	85.6±6.4	80.1± .7
Ovariectomized control	4	7	85.5± 7.5	19.7±1.7	77.7± .4
EB:.2 μg/day	5	5	285.2±31.6	55.6±6.2	80.5± .2
Eb:.2 μg/day+R:15 GPU/day	6	5	403.0±40.5	71.4±8.7	82.3± .3
EB:1 μg/day	7	7	369.2±20.8	74.1±4.5	79.9± .3
EB:1 μg/day+R:15 GPU/day	8	9	407.4±22.1	78.1±3.9	80.8± .2
EB:1 μg/day+R:15 GPU/day +P:1 mg./day	9	9	446.8±23.5	89.6±4.2	79.8± .5
EB:1 μg/day+P:1 mg./day	10	7	299.8±34.3	60.1±7.0	79.9± .2
P:1 mg./day	11	11	119.0±11.8	25.5±2.4	78.6± .4
P:5 mg./day	12	6	120.8±10.2	25.1±2.3	79.1± .4
R:15 GPU/day	13	9	133.1±21.2	28.2±3.6	78.4± .6

EB = Estradiol Benzoate; R = Relaxin; P = Progesterone.

Groups tested	P Value		
	Wet wt.	Dry wt.	Water content
1– 2	n.s.	n.s.	0.05
1– 3	0.001	0.001	n.s.
2– 3	0.01	0.01	n.s.
1– 4	0.01	0.05	n.s.
2–13	n.s.	n.s.	0.05
3– 8	n.s.	n.s.	n.s.
4– 5	0.001	0.001	0.001
5– 6	0.05	n.s.	0.01
5– 7	0.05	0.05	n.s.
4– 7	0.001	0.001	0.01
4– 8	0.001	0.001	0.001
7– 8	n.s.	n.s.	0.05
7– 9	0.05	0.05	n.s.
7–10	n.s.	n.s.	n.s.
8– 9	n.s.	n.s.*	n.s.*
8–10	0.02	0.05	0.05
9–10	0.01	0.001	n.s.
10– 6	n.s.*	n.s.	0.001
4–11	n.s.*	n.s.	n.s.
4–13	n.s.*	n.s.*	n.s.
11–12	n.s.	n.s.	n.s.

n.s. = none significant; * = $P < 0.1$.

weight in the lower dose (0.2 μg), but it was masked by EB in the higher dose (1 μg) (Table 2).

Administration of combination of EB, P and R caused a significant increase in wet and dry weight of uterus over those of EB injected animals. A significant difference in uterine weight between EB plus R and combination of 3 hormones was not observed.

In intact groups in first experiment, administration of combination of EB and R caused a significantly higher uterine water content in comparison to EB treatment but without significant difference between 2 doses of R. No significant differences were observed among the castrate groups.

In the second experiment, both doses of EB raised water content significantly in the uterus compared to castrate control. Relaxin enhanced, significantly, effect of EB on uterine water content in both groups receiving estrogen.

A slight decrease in uterine water content in rats receiving combination of 3 ovarian hormones was observed in comparison with that of animals receiving the combination of EB and R. Neither R nor P alone increased water content of uterus.

DISCUSSION

It has been shown in mouse (9, 10) and rat (11) that injection of EB and R stimulated growth of lobule-alveolar system of mammary gland comparable to that stimulated by EB and P during normal pregnancy, In conjunction with this latter study (rat) observation on influence of EB and R on wet and dry uterine weight was made.

In intact mature rats significant increases in uterine weight were observed with EB and R, whereas in ovariectomized animals the increases were less significant. The reduced effect in ovariectomized animals may have been due to marked involution of the uterus which preceded treatment period. Marked influence on uterine growth of EB and R in intact rats may have been due, in part, to endogenous progesterone. This is suggested by the results of the second experiment, in which immediate administration after castration of EB or of EB plus R prevented uterine involution, and exogenous P augmented the effect of combination of EB and R.

Present data indicate that estrogen stimulates an increased wet and dry weight of uterus and restores involuted uterus following ovariectomy. Relaxin slightly augments uterine growth-promoting effect of estrogen, but induces a significant hydration of the uterus. Progesterone reduced effect of estrogen on uterine growth and had no appreciable effect on uterine growth. However, P enhanced action of EB and R, and increased uterine weight with rather slight decrease in hydration due to increase of dry weight.

It is concluded that endogenous P is involved, in part, in increased response of the uterus in the intact rat. Results of present experiment confirm previous reports (4, 6). As maximum uterine hydration due to R was observed at 6 hours following injection (6, 7), 6-hours assay would be required for a more elevated hydration.

It appears that EB and R separately, or in conjunction with P, have the capacity to stimulate growth in 2 tissues of the reproductive system, namely, the mammary glands (10) and uterus during injection periods corresponding to the first half of pregnancy.

HIROSHI WADA[3] AND CHARLES W. TURNER

Department of Dairy Husbandry
University of Missouri
Columbia, Missouri

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[3] Research Scholar, Ministry of Education, Japan, and Medical Fellow of Population Council. Present address: College of Agriculture, Univ. of Okayama, Japan. This investigation supported in part by grant from Am. Cancer Soc.

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EFFECT OF VASOPRESSIN ON SODIUM TRANSFER BY RAT COLON *IN VITRO*[1]

ABSTRACT

Everted segments of rat colon incubated in Krebs-Ringer bicarbonate containing glucose consistently show increased net transfer of sodium from mucosal to serosal fluid when Pitressin is present in the serosal fluid. When Pitressin is present only in the mucosal fluid, this effect is not observed.

Blickenstaff (1) has reported an increase in water absorption from fistulas of jejunum in dogs after Pitressin injection, and Ussing (2) observed that the drug produced a transient increase in the short circuit current (active Na transport) across the isolated mucosa of the guinea pig cecum. The latter has also shown that Pitressin increases active transfer of sodium and flow of water across frog skin (3). Leaf (4) has reported a similar effect of Pitressin on active sodium transfer and water movement across isolated toad bladder.

An *in vitro* method for study of sodium absorption by segments of rat intestine has been developed in this laboratory, and it seemed of interest to study the effect of vasopressin on this preparation.

METHODS AND MATERIALS

The preparation is a modification of the method of Wilson and Wiseman (5). Male Sprague-Dawley rats weighing 230–260 gm. were used. The animals were fasted for 20 hours, then given 6 cc. of 12% ethanol/100 gm. body weight by stomach tube under light ether anesthesia. Forty minutes later, the abdomen was opened and about the first 7 cm. of large gut separated from the rest of the tract and flushed thoroughly with warm 0.9% NaCl, while still joined to the mesentery. In earlier experiments we found that it is essential to minimize the period of anoxia between removal of the segment and placement in the incubation medium. After flushing, the segment was cut away from the mesentery, rinsed in warm 0.9% NaCl and everted with a glass rod. One end was tied off, 2.0 cc. of buffer placed in the serosal (inside) compartment using a blunted needle and 5 cc. syringe, and the remaining end tied off and the segment placed in a 110×65 mm. crystalizing dish containing 50 cc. of buffer already being gassed with 95% O_2—5% CO_2 and shaking at 125 oscillations/minute in a Dubnof incubator. The buffer, which was used for both serosal and mucosal (outside) fluids, was a Krebs-Ringer bicarbonate having 135 mM Na, glucose 200 mg.% and about 0.001 μc

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of radiosodium 22/100 cc. Each experiment included a segment from each of four animals, two serving as controls and two as experimentals. In the first series of experiments vasopressin was present only in the serosal fluid and in the second only in the mucosal fluid. The concentration in all cases was of 0.1 unit/cc.

After three hours of incubation the segments were blotted, opened and drained into weighed sample tubes, and the final serosal volume of fluid determined by a second weighing. In spite of the long period of incubation, segments do not slough mucosa when blotted. Radiosodium was counted in a scintillation well. The specific activity of the labeled buffer used for incubation medium and injection of the segments was determined by counting a measured volume of the buffer and calculating counts/10 minutes/μg total Na. This value and counts obtained from the serosal fluid of incubated segments were used to calculate the amount of sodium absorbed into the serosal compartment. Segments were dried in an oven, and sodium absorption in μg Na/mg. tissue dry weight determined. Commercial vasopressin (Pitressin, Parke, Davis) was employed in all experiments.

RESULTS

Segments of colon incubated under the conditions described showed a net transfer of fluid and sodium from mucosal to serosal compartment (absorption). Sodium was absorbed in excess of water, and the concentration of this ion in the serosal fluid increased. In each case in which hormones were present in the serosal fluid, increased absorption of sodium occurred, and final concentrations of sodium were higher in the serosal fluid of treated segments (Table 1). In the two experiments in which Pitressin was present only

TABLE 1. EFFECT OF PITRESSIN IN THE SEROSAL FLUID ON SODIUM ABSORPTION BY RAT COLON

Exp. No.	Sodium absorption, micrograms/mg. dry tissue		Final concentration, serosal fluid sodium. Initial concentration 135 mM.	
	Control	Pitressin	Control	Pitressin
1	12.3	—	172	—
	8.2	13.5	160	163
2	2.7	11.2	153	165
	11.0	22.8	158	
3	12.1	21.2	171	172
	—	17.5	—	169
4	11.1	12.5	173	190
	12.2	14.0	162	188
	$\bar{x}$ 9.9	16.1	$\bar{x}$ 164	173
	S_x = 1.53		S_x = 3.50	
	t = 4.05		t = 2.57	

in the mucosal fluid, no consistent effect of the hormones on sodium absorption was observed (Table 2.). Segments from all animals also absorbed small amounts of water into the serosal compartment (less than one cc.), but no effect of Pitressin on water absorption was observed.

DISCUSSION

Parsons and Paterson (6) recently reported studies of an *in vitro* preparation of rat colonic mucosa which showed solute absorption in excess of water from "isotonic" mucosal fluid, as was observed in our preparation. The effect of Pitressin on sodium transport observed here is similar to that reported by Ussing (3) in frog skin and Leaf (4) in toad bladder. Leaf and his associates have also found that Pitressin accelerates the passive movement of water and urea across the wall of toad bladder from mucosal to serosal surfaces, and have concluded that neurohypophyseal hormones increase the

TABLE 2. SODIUM ABSORPTION BY SEGMENTS OF RAT COLON WITH AND WITHOUT PITRESSIN IN THE MUCOSAL FLUID

Exp. No.	Sodium absorption, micrograms/mg.		Final concentration serosal fluid sodium. Initial concentration, 135 mM	
	Control	Pitressin	Control	Pitressin
1	10.9	5.5	168	163
	7.9	10.0	163	164
2	12.2	8.0	170	160
	5.7	11.1	158	159

permeability of a mucosal diffusion barrier to water, sodium and urea. Increased active sodium transport is thought to be secondary to this increase in permeability (4). These investigators also found that Pitressin exerts its effects on permeability only when present in the fluid bathing the serosal surface of the bladder. We find a similar requirement for the effect of Pitressin on sodium absorption by the rat colon *in vitro*. Curran and Schwartz (7) have shown that water absorption by rat colon *in vivo* is a passive process, dependent on solute absorption. It may be that absorption of water into the serosal compartment of our segments is limited by their elastic walls. Thus when the serosal volume reaches a certain level, water is forced out as rapidly as it enters. Absorption of sodium continues, however, and the cation accumulates in the serosal compartment.

It must be emphasized that the amounts of Pitressin used in these experiments were large (0.1 unit/cc.). Nevertheless, it is tempting to think that anti-diuretic hormone exerts effects on intestine similar to those on amphibian skin and bladder, and the kidney.

KENNETH A. AULSEBROOK

Department of Physiology
School of Medicine
University of Arkansas
Little Rock, Arkansas

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RESPONSE OF THE THYROIDAL IODIDE PUMP IN HYPOPHYSECTOMIZED RATS ON A LOW IODINE DIET TO THE PLASMA OF GOITROUS RATS[1]

ABSTRACT

The thyroid/serum radioiodide concentration ratio (T/S) of hypophysectomized rats fed a Remington diet rose in response to the injection of 3 ml. of plasma from normal or triiodothyronine treated rats only if at least 1.5 mU of TSH was added to it. The same amount of plasma from rats with propylthiouracil-induced goiter, however, raised the T/S without the addition of TSH. The contradictory published findings concerning the thyrotrophic activity of the serum of normal and goitrous rats are briefly discussed.

Low iodine intake enhances the response of the thyroid/serum radioiodide concentration ratio (T/S) of hypophysectomized rats to large doses of TSH (1, 2). The smallest amount of TSH which was shown significantly to raise the T/S of hypophysectomized rats fed adequate amounts of iodine was 5 (3) or 10 (4) mU. This report demonstrates that iodine deficiency increases the sensitivity of the T/S response in hypophysectomized rats so that 1.5 mU. of TSH or the roughly equivalent thyrotrophic activity of 3 ml. of plasma from goitrous rats can be detected.

MATERIALS AND METHODS

Plasma was obtained by withdrawing (into heparinized syringes) and pooling blood from the aorta of a) normal rats weighting 250–300 gm., b) rats treated with 10 μg of L-triiodothyronine sodium (T3) for 22 days (body weight 200–250 gm.), and c) rats fed propylthiouracil (PTU) in the diet (0.03%) for 22 days (body weight 200–250 gm.). All these rats were males of the Holtzman strain and received Rockland diet. TSH (Armour)[2] was mixed with portions of the pooled plasma from the rats in groups a) and b) immediately after its separation by centrifugation to yield the amounts per 3 ml. shown in Figure 1. The recipients were hypophysectomized male Sprague-Dawley rats obtained from the Hormone Assay Laboratories, Chicago. They were started on a regimen of Low Iodine Test Diet (Nutritional Biochemicals, Cleveland) 5–7 days postoperatively. This diet was given for 15 days. During the last 4 days each of these rats received 2 mg. of cortisone acetate subcutaneously to bolster their resistance and diminish possible adverse reactions to injected plasma. Cortisone was found not to interfere with the T/S response of hypophysectomized rats to TSH (5). Twenty-four hours before the rats were killed they were injected intraperitoneally with 3 ml. of saline or of plasma freshly separated from donor blood (with or without TSH added). T/S determinations were performed by the method of VanderLaan and Greer (6).

RESULTS

Figure 1 shows the response of the T/S to plasma of goitrous rats and its failure to rise under the effect of normal plasma or that of T3 treated rats unless at least 1.5 mU of TSH was added to it.

DISCUSSION

The method used in this study was sensitive enough to detect endogenous thyrotrophic activity in 3 ml. of plasma from rats fed 0.03% PTU in the diet for 22 days, or 1.5 mU of TSH added to the plasma of T3 treated rats which presumably had no endogenous TSH activity and may even have contained a substance or substances antagonistic to TSH (7).

McKenzie (8) has recently reviewed the literature concerning TSH content of human serum. We are not aware of a comparable compilation of the much scantier information regarding rat serum or plasma.

The levels of thyrotrophic activity reported for normal rat serum vary considerably. Jagiello and McKenzie (9) found no activity in 0.5 ml. with the mouse thyroid I^{131}

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[2] Generously supplied by Dr. J. D. Fisher. Stated potency 0.9 U.S.P.U./mg.

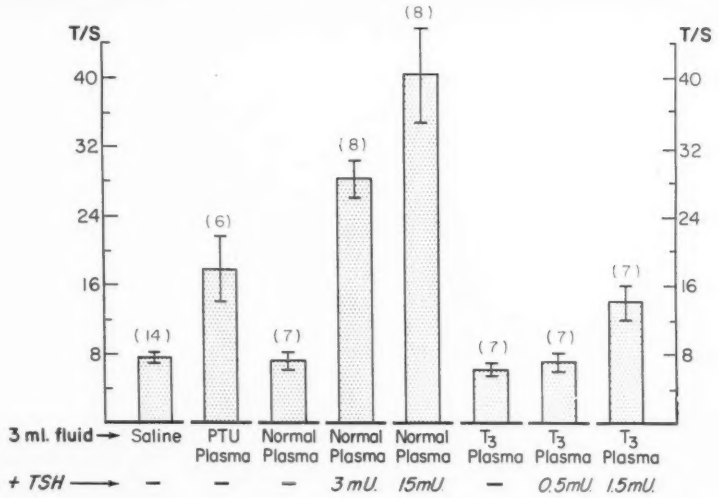

FIG. 1. Effect of plasma from goitrous rats or from normal rats and T3 treated rats (with or without TSH added) on the thyroid/serum radioiodide concentration ratio (T/S) of hypophysectomized rats. Vertical bars; means; vertical lines: ±standard errors of the mean. Number of recipients in parentheses. Statistical significance (bars counted from left to right): 1 vs. 3, 6, 7 $P > 0.1$; 2, 4, 5, 8 vs. 1, 3, 6, 7 $P < 0.01$; 2 vs. 8 $P > 0.1$; 2 vs. 4 $P = 0.03 - 0.04$; vs. 5 $P = 0.06 - 0.07$; 2 vs. 5 $P = 0.01 - 0.02$; 4, 5 vs 8 $P < 0.01$.

release method (10) which is said to demonstrate as little as 0.025 mU. Our method showed no activity in 3 ml. plasma. Del Conte and Stux (11), who used a technique based on cytologic changes elicited in the guinea pig thyroid, found 0.004–0.012 mU/ml.[3] On the other hand, the stasis tadpole method of D'Angelo (12, 13, 14 and personal communication) reproducibly demonstrates concentrations of 0.6–1.0 mU/ml. Such amounts should have raised the T/S significantly in our recipient rats.

The reported observations for serum of rats made goitrous with thiocarbonamides are also contradictory. Our rough estimate of Jagiello and McKenzie's (9) results is based on (10), since they assayed no standard preparation concomitantly, and suggests about 0.4 mU/ml. in the serum of rats which had received a diet containing 0.03% PTU for 13 days. The results of this study indicate that in rats fed 0.03% PTU for 22 days the plasma TSH level is 0.5–1.0 mU/ml., and probably closer to the lower limit. Del Conte and Stux (11) estimated 2.0–6.0 mU/ml.[3] in the serum of rats given 10–20 mg. thiourea daily for 30 days. D'Angelo reported about 1.3 mU/ml. after 0.05% PTU in the diet for 18 days in one study (12) and 1.6 mU/ml. in the serum of rats which were on 0.1% PTU in the diet for 30 days. It is remarkable that according to Del Conte and Stux (11) serum TSH rose 500-fold under the effect of a relatively mild goitrogen, whereas in the experience of D'Angelo one month's treatment with the potent PTU

[3] McKenzie (7) has pointed out the uncertainties regarding the potency of the Junkmann-Schoeller (J.S.) unit vs. the U.S.P. unit used throughout this note. He considers 1 U.S.P.U. = 10–12 J.S.U. the most reliable estimate. D'Angelo, however, used a standard in his earlier study (11) which was said to contain 8–10 J.S.U./mg., but was later found to have a potency of 2 U.S.P.U./mg. (personal communication). Hence for the results of Del Conte and Stux (10), which were expressed in J. S.U., a range is given.

elevated it only 1.6-fold (13). Methods which fail to show thyrotrophic activity in normal rat plasma or serum, such as that of McKenzie (10) and the one used in this study, obviously cannot contribute to the resolution of this enormous discrepancy.

It is evident that the question of the level of thyrotrophic activity in normal rat serum and that of goitrous rats is far from being settled. Further work is needed on identical sera with different methods sensitive enough to permit not only demonstration of TSH in normal rat serum but also 2+2 assays against simultaneously run standards to make statistical evaluation of the results feasible. The possibility that substances antagonistic to TSH lower the demonstrable activity in some assays but not in others may then emerge. Until such comparative assays are reliably done, the discrepancies compounded and reviewed in this note will remain completely inexplicable.

D. K. GRANNER,[4] S. J. CURTIS[5] AND N. S. HALMI

Department of Anatomy
State University of Iowa
Iowa City, Iowa

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[4] NIH predoctoral trainee

[5] NIH summer medical student research fellow.

EFFECTS OF THYROXINE AND TRIIODOTHYROPROPIONIC ACID ON GROWTH OF THIOURACIL-TREATED RATS

ABSTRACT

Weanling rats treated chronically with thiouracil in the diet showed significant depression of growth rate by the end of 2 weeks and virtually complete cessation by 6–7 weeks. It was possible to reinstate growth with minute fractions of a daily, non-calorigenic dose of L-thyroxine or 3,5,3′-triiodothyropropionic acid. Perceptible growth was obtained with a minimum of 0.04 μg/100 gm. b.wt./day of either compound. The extreme sensitivity of growth to minute doses suggests a useful method of selecting compounds for veterinary and human clinical trial.

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Recently, Evans, *et al.* (1) demonstrated quantitative differences in L-T4 requirements for a number of biological responses within the same rat. They administered L-thyroxine (L-T4) daily, for 96 days, to rats thyroidectomized at 28 days age. We have also observed and reported (2, 3) different ratios of effectiveness for L-T4 and several of its analogues for different biological endpoints in intact rats, with and without concomitant thiouracil (TU) treatment. Of particular interest is Evans' observation (1) that significant growth could be reinstated with relatively minute non-calorigenic daily doses of L-T4 in rats thyroidectomized at an early age. We have obtained similar results with animals whose body weight increase was stopped at an early age by chronic TU treatment.

MATERIALS AND METHODS

Thirty-nine male rats, 23 days of age on day 0 (Fig. 1), were divided into 3 groups of 13 each. One group served as normal controls throughout the experiment. The other 2 groups were allowed to eat powdered Rockland Rat Diet containing either 0.2% or 0.3% TU until day 23. The growth-inhibiting effects (using body wt. as criterion of growth) at these concentrations appeared to be equivocal during the first three weeks of TU treatment. Thereafter all of the rats received 0.2% TU. On day 33 the 21 surviving TU-treated rats were divided into 3 groups of 7 each. One group served as TU controls throughout the balance of the experiment. The other two groups received several courses of daily subcutaneous injec-

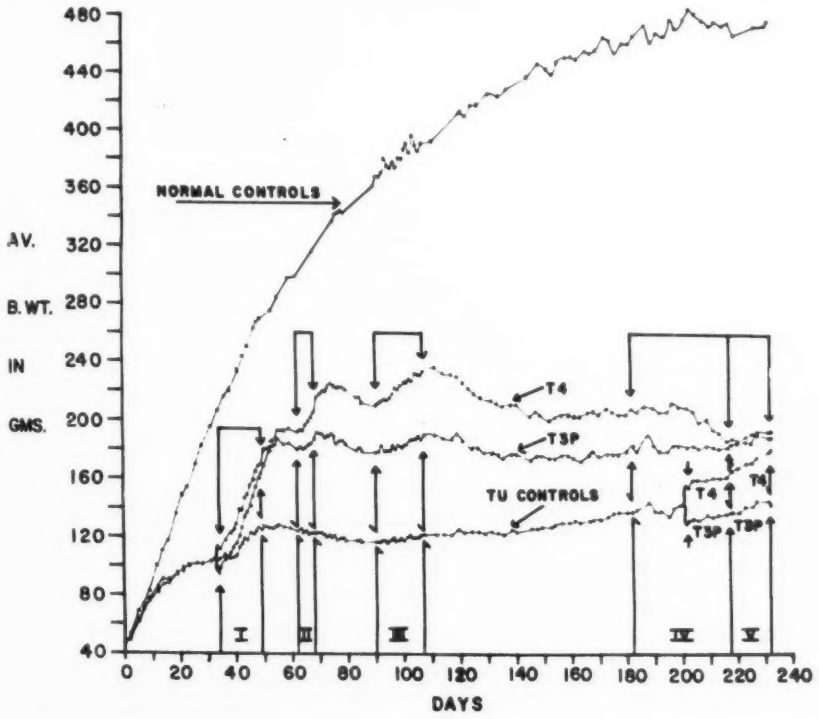

FIG. 1. Effect of thiouracil (TU) fed chronically in the diet and subcutaneous injections of L-thyroxine (L-T4) or 3,5,3′-triiodothyropropionic acid (T3P) on body weight of male rats. Doses of L-T4 and T3P during Periods I, II, III, IV and V were 4.0, 0.8, 0.2, 0.02 and 0.04 g./100 gm. b.wt./day, respectively.

tions of L-T4 sodium pentahydrate or 3,5,3′-triiodothyropropionic acid (T3P) at various dosages and for varying lengths of time depending on the body weight responses obtained. All rats were allowed diets and tap water *ad libitum* and body weights were measured regularly. Injectable alcoholic-alkaline solutions of L-T4 and T3P were prepared and stored cold as previously described (2, 3).

RESULTS AND DISCUSSION

Figure 1 demonstrates that chronic treatment of weanling rats with TU in the diet caused significant retardation of growth by day 14. Body weight gain virtually ceased after 6–7 weeks (day 42–49) of goitrogen treatment as shown by the TU controls. The first course of L-T4 and T3P injections was from days 33–47 at doses calculated to be biologically equivalent and non-calorigenic in chronically TU treated rats (3). Growth rates were immediately accelerated to normal by both compounds and maximum gains were reached about 7 days after the last injection. L-T4 appeared to have slightly more activity than T3P. Subsequent courses of L-T4 and T3P at 0.8 and 0.2 μg/100 gm. b.wt./day (1/5 and 1/20 of the dose used in the first course of treatment) produced additional, although proportionately less, growth. However, given sufficient time between courses of treatment, the apparent growth at these lower doses was partly or completely reversed in the interim. Following the third course of treatment with L-T4 and T3P no injections were given from days 108 to 182. During the first half of this period the previously stimulated groups lost an average of 20–35 gm. body weight. Since weight was the sole criterion of response, the apparent growth observed earlier at doses less than 4 μg could have been largely due to increased protein and fat deposition (4) with only slight increases in linear growth (body length).

When L-T4 and T3P were injected at a dose of 0.02 μg./100 gm/day, for 35 days, beginning on day 182, growth stimulation was not obtained. In fact, the L-T4 group lost about 20 gm. during the latter half of this dose period (202–217 days). At this time, (202–217 days), it will be noted that the TU control group was utilized to obtain reinforcing information regarding response or no-response to such minute doses. Consequently, it was observed that while none of the TU-treated groups responded to daily doses of 0.02 μg L-T4 or T3P, all of the groups appeared to give a very slight response to 0.04 μg of either compound during the last injection period (days 217–232). Thus, it appears that under our test conditions, the minimum daily dose of L-T4 needed to obtain a measurable increase in growth is about 0.04 μg. Evans, *et al.* (1) suggests 0.05 μg L-T4, or less, as the minimum. In addition T3P appears to be at least half as potent as L-T4 when both compounds are given subcutaneously. This is in good agreement with results obtained in calorigenic and goiter-prevention comparisons of these compounds in TU treated rats (3). However, when given in the diet T3P has been found to have 2–4 times the calorigenic activity of L-T4 in rats (5).

The work of Evans, *et al.* (1) and the present report demonstrates that rats whose growth has been stunted by surgical thyroidectomy (1) or chronic TU treatment (this report) are extremely sensitive to minute, non-calorigenic doses of L-T4 and T3P as shown by rapid reinstatement of growth. When the parameters of this test system are delineated it may afford a convenient method for comparing the relative activities of L-T4 analogues. It would be of interest, and possibly of therapeutic implications, to determine the ratio of *maximum non-calorigenic dose to minimum dose for restoration of normal growth*. Analogues with the highest ratios would be selected for clinical trial, e.g., in accelerating growth of domesticated animals and retarded children.

NEIL R. STASILLI, ROBERT L. KROC AND PAUL J. NEMITH
Warner-Lambert Research Institute
Morris Plains
New Jersey

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THE EFFECT OF A HYPOTHALAMIC EXTRACT ON THE PLASMA LUTEINIZING HORMONE (LH) ACTIVITY OF THE ESTROGENIZED, OVARIECTOMIZED RAT[1]

ABSTRACT

The LH activity of rat plasma was estimated by the ovarian ascorbic acid depletion method. Significant LH activity was found in plasma from rats which had been ovariectomized for 1–4 months. A single subcutaneous injection of 1.0 μg of estradiol benzoate produced a marked decrease in this activity on assay 3 days later. The LH activity in plasma of such estrogen-treated, ovariectomized rats was significantly elevated within 10 minutes after the intravenous injection of an acidic extract of rat stalk-median eminence tissue, whereas injection of extract from cerebral cortex was without significant effect. It is suggested that stalk-median eminence tissue contains a specific LH-releasing factor.

An acidic extract of rat hypothalamic tissue prepared from the stalk-median emience region possesses significant LH-releasing activity as measured by ovarian ascorbic acid depletion in immature rats pretreated with gonadotophins (1). In this test the injected material is assumed to act upon the hypophysis of the assay animal, to cause a secretion of LH, which, in turn, evokes ascorbic acid depletion in the ovaries. The effect obtained was a small one, possibly because of the small amount of LH found in the hypophysis of these immature assay animals. Therefore, it appeared of interest to test the LH-releasing activity of these extracts using another assay method.

To provide a more sensitive assay, another step was introduced into the assay, a step employing ovariectomized, adult rats treated with estrogen. Such animals possess elevated quantities of LH in both plasma and pituitary galands (2, 3), and treatment of these ovariectomized rats with estrogen can markedly decrease the plasma LH activity (4). The present study reports the effect of intravenous injection of crude extracts of the stalk-median eminence region of rats on the LH activity of plasma of the estrogen-treated, ovariectomized rat. This LH activity was determined by the ovarian ascorbic acid depletion test first described by Parlow (1, 5).

METHODS

All rats were of the Sherman strain. Acidic extracts of rat stalk-median eminence tissue or cerebral cortex were prepared from the brains of large male rats (~400 gm. in body weight) as previously described (1). These extracts were injected into ovariectomized rats to see if they altered the plasma LH activity.

The ovariectomized rats were adult females which had been ovariectomized for one to four months. To provide a control value for the LH content of ovariectomized rat plasma, some of the ovariectomized rats were bled while anesthetized with ether, and their plasma assayed for LH activity. The remaining ovariectomized rats were injected subcutaneously 68–72 hours prior to use with a single dose of 1.0 μg estradiol benzoate dissolved in 0.1 ml. of sesame oil. Vaginal smears of all these rats were in the estrous stage at the time of use. One group of these ovariectomized, estrogen-treated rats was anesthetized with ether and bled.

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The plasma from these rats was assayed for LH activity to determine the effect of estrogen treatment on plasma LH activity of the ovariectomized rat. The other group of estrogen-treated, ovariectomized rats was also anesthetized with ether, and then injected intravenously during a period of one minute with either stalk-median eminence extract derived from two rat hypothalami, or cerebral cortex extract derived from an equivalent weight of tissue. This group of ovariectomized, estrogen treated rats was kept anesthetized with ether, and was bled ten minutes after beginning injection of the extract.

All ovariectomized rats were bled from the external jugular vein, and four to six ml. of blood were collected in a heparinized syringe during a period of one to three minutes. Blood from four donors subjected to the same treatment was pooled and the plasma separated by centrifugation. Each assay rat received two ml. of plasma and five animals were used for each pool of blood. Many pools of blood from ovariectomized rats, three from ovariectomized rats injected with estrogen alone, three from those injected with estrogen plus cerebral cortex, and four from ovariectomized, estrogen-treated animals injected with stalk-median eminence extract were tested.

For assay of LH activity, one ovary of each assay rat was first removed for a control value of ovarian ascorbic acid. Then two ml. of plasma were injected intravenously over a period of 45–60 seconds. One hour later the second ovary was removed, and its ascorbic acid concentration determined. The results have been expressed as the percentage depletion (decrease) in ascorbic acid concentration of the second ovary as compared to the control value obtained in the first gland.

RESULTS AND DISCUSSION

By the test described, plasma of ovariectomized rats contained a significant amount of LH, and this amount could be lowered by administration of estrogen ($P < .001$) (Table 1). When it had been so lowered, the injection of an extract of cerebral cortex

TABLE 1. OVARIAN ASCORBIC ACID DEPLETION INDUCED BY PLASMA FROM OVARIECTOMIZED RATS

	Ovariectomized	Estrogen-treated, ovariectomized		
		No extract	Cerebral cortex extract	Stalk-median eminence extract
		1 ±2.2 (5)*	12.8±2.6 (5)	15.4±2.5 (5)
		4.7±2.4 (5)	4.0±1.0 (5)	17.5±4.0 (5)
		8.3±2.3 (5)	7.3±1.5 (5)	18.6±2.0 (5)
				19.1±2.5 (5)
Overall mean	15.3±.7 (53)[1]	4.7±1.5 (15)	8.0±1.6 (15)	17.7±1.3 (20)

* These values represent the mean and standard error of the mean obtained on assay of each pool of plasma. The overall mean is obtained by combining all assays for a given treatment. The numbers in parentheses represent the number of assay rats tested.

[1] Because of the large number of pools of plasma assayed in this group, the values for individual pools have been omitted.

failed to elevate it significantly ($P < .2$). The injection of an extract of hypothalamus by contrast, did evoke a significant increase in plasma LH activity ($P < .001$). The activity present in plasma ten minutes after injection of stalk-median eminence extract into these estrogenized, ovarectomized rats was similar to that found in the ovariectomized controls.

These results could hardly be caused by LH contamination of the extracts since no detectable LH-like activity was found on assay in hypophysectomized assay rats at the dose employed here (1). Furthermore as a result of injection into and sampling from a donor rat, any LH-like activity present in the extract should be markedly diluted at the time of injection of the plasma sample into the assay rats. Consequently, it appears that injection of stalk-median eminence extract evoked a significant secretion of LH in the estrogenized, ovariectomized rat, confirming the results obtained previously by direct injection of the extract into the assay animals (1).

At that time, evidence was presented suggesting that the LH-releasing activity of these extracts was not accounted for by vasopressin or a variety of other pharmacologically active agents present in hypothalamic tissue (1). It is suggested that this LH-releasing activity may be caused by a specific LH-releasing factor present in the extract. Future work must determine if this material is a polypeptide similar to the corticotrophin-releasing factor already shown to be present in stalk-median eminence tissue (6, 7).

S. M. McCann and S. Taleisnik

Department of Physiology
University of Pennsylvania
School of Medicine
Philadelphia, Pennsylvania

Acknowledgment

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PAPER ELECTROPHORETIC DEMONSTRATION OF THYROXINE-BINDING PREALBUMIN FRACTION IN SERUM[1]

ABSTRACT

Several amine buffer systems have been utilized to demonstrate the presence of a thyroxine-binding prealbumin fraction in human serum.

The presence of a thyroxine-binding prealbumin (TBPA) fraction in serum has been demonstrated by Ingbar (1) using paper electrophoresis in tris (hydroxymethylaminomethane)-maleate buffer. TBPA has also been shown using reverse-flow electrophoresis in ammonium carbonate buffer (2). In more recent reports (3, 4) the presence of TBPA has been observed in borate, phosphate, bicarbonate, aminediol, glycine and alanine buffers. We wish to describe several amine buffer systems that have been particularly effective in demonstrating the presence of TBPA in serum.

METHODS

Electrophoresis of serum was carried out with a Durrum type paper electrophoresis cell (Spinco). The migration of thyroxine-binding fractions was studied by adding I^{131}-labeled L-thyroxine in varying amounts to human serum albumin and determining the zones of

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[1] Supported by grants from the National Institutes of Health and the American Cancer Society, and by a research contract with the U. S. Atomic Energy Commission.

radioactivity on the paper strips with a Nuclear-Chicago strip scanner, as well as by developing radioautographs. Electrophoresis was carried out for 18–20 hours at 60 volts and 6 m.a. except that in the case of methylamine-maleate buffer, 32 volts and 2 m.a. were used. All buffers were adjusted to pH 8.6 with sodium hydroxide. The ionic strength, $\Gamma/2$, approximated 0.15 in all instances.

RESULTS

The following buffer systems gave distinct separation of a TBPA fraction: 0.2 M ammonium hydroxide—0.05 M maleate; 0.2 M glycine—0.05 maleate; 0.20 M glycine —0.13 M acetic acid; 0.2 M methylamine—0.05 M maleate.

The best separations of TBPA were observed with glycine-maleate and glycine-acetate buffers. Figure 1 shows radioautographs of electrophoresis in glycine-acetate buffer. With methylamine-maleate buffer very good separations of TBPA were obtained although the TBPA zone was diffuse.

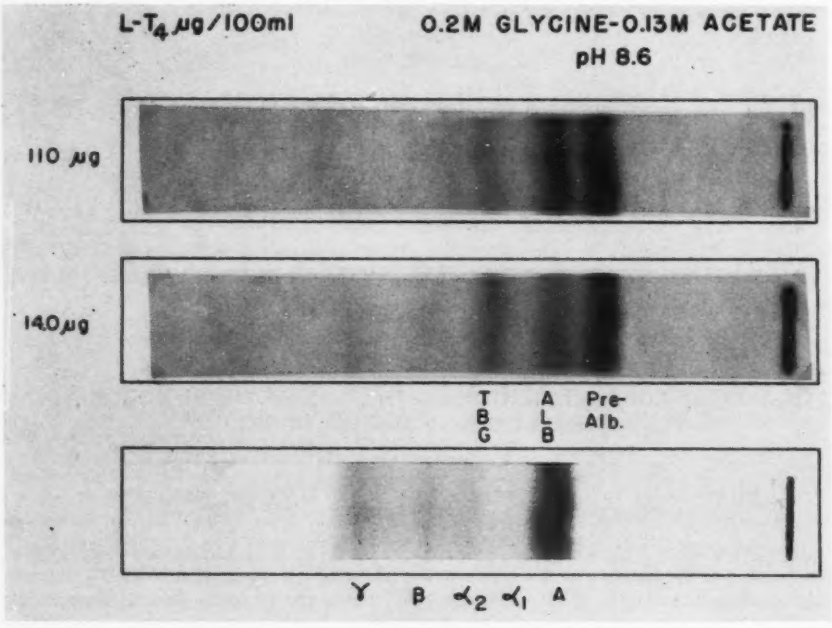

FIG. 1. The two upper strips are radioautographs with added carrier thyroxine, as indicated by concentrations at left. The lowest strip is a bromphenol blue stain of the serum proteins. The vertical lines at the right of all three strips are markers for alignment.

DISCUSSION

These results provide further support for the presence of a thyroxine-binding prealbumin fraction in human serum. It is hoped that this prealbumin may be obtained in purer form for more quantitative studies. For this reason, it has seemed important to study the resolving powers of various buffer systems for future preparative work. The physiological role of this fraction in hormone transport remains to be elucidated.

Acknowledgment

We wish to thank Mr. Robert Danziger for valuable technical assistance.

KENNETH STERLING AND MILTON TABACHNICK

Department of Internal Medicine
N. Y. State Psychiatric Institute
and Department of Psychiatry
Columbia University College of Physicians
and Surgeons
New York, N.Y.

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THE ENDOCRINE SOCIETY

1962 AWARDS

Fred Conrad Koch Award

A substantial legacy has been bequeathed the Society by the late Elizabeth Koch for the purpose of establishing the Fred Conrad Koch Memorial Fund in memory of her late husband, distinguished service professor of physiological chemistry at the University of Chicago and pioneer in the isolation of the androgens. This is to be the highest honor of the Endocrine Society and is to be represented by a medal that is to be known as the Koch Medal of The Endocrine Society. The medal and honorarium of $3,500 is to be given annually to an individual for work of special distinction in endocrinology.

The recipient shall be chosen from nominations presented by members of the Society and is limited to citizens of the United States and Canada. This award will replace the Medal of the Endocrine Society which was established in 1954 and presented to Dr. Carl Moore in 1955, Dr. Frederick L. Hisaw in 1956, Dr. Joseph C. Aub in 1957, Dr. I. L. Chaikoff in 1958, Dr. Wilbur W. Swingle in 1959 and Dr. Emil Witschi in 1960. The Endocrine Medal replaced the E. R. Squibb Award which was formerly the highest honor bestowed by the Society. Past recipients of the Squibb Award were Dr. George W. Corner in 1940, Dr. Philip E. Smith in 1941, Dr. Fred C. Koch, 1942, no award was given in 1943, Dr. E. A. Doisy in 1944, Dr. E. C. Kendall in 1945, Dr. Carl O. Hartman in 1946, Drs. Carl F. and Gerty T. Cori in 1947, Dr. Fuller Albright in 1948, Dr. Herbert Evans in 1949, Dr. C. N. H. Long in 1950, Dr. J. B. Collip in 1951, Dr. James H. Means in 1952 and Dr. David Marine in 1953.

The Ciba Award

The Ciba Award, to recognize the meritorious accomplishments of an investigator who has not attained his 36th birthday by June 1st of the year the award is presented, in the field of clinical or preclinical endocrinology, was established in 1942, but no recipient was selected in 1942 or 1943. In 1944 the Award was presented to Dr. E. B. Astwood, 1945 Dr. Jane A. Russell, 1946 Dr. Martin M. Hoffman, 1947 Dr. Choh Hao Li, 1948 Dr. Carl Heller, 1949 Dr. George Sayers, 1950 Dr. Oscar M. Hechter, 1951 Dr. Albert Segaloff, 1952 Dr. Seymour Lieberman, 1953 Dr. Sidney Roberts and Dr. Clara Szego (Mrs. Roberts), 1954 Dr. Isadore M. Rosenberg, 1955 Dr. Jack Gross, 1956 Dr. Alfred M. Bongiovanni, 1957 Dr. Nicholas S. Halmi, 1958 Dr. Monte Arnold Greer, 1959 Dr. Gordon L. Farrell and 1960 Dr. Don H. Nelson. Prior to 1952 the Award was $1,200. It has now been increased to $2,500.

The Ayerst and the Squibb Fellowships

The Ayerst Fellowship was established in 1947 and the Squibb Fellowship in 1956. They are designed to assist men or women of exceptional promise in furthering their advancement towards a career in endocrinology. Each Fellowship is awarded on alternate years and the stipend, which will not exceed $7,500 may be divided into two Fellowships in varying amounts in accordance with the qualifications of the appointees. Individuals possessing the M.D. or Ph.D. degree, or candidates for either of these degrees, are eligible for appointment.

Applicants must submit the following information:

1. Evidence of scientific ability as attested by studies completed or in progress.
2. Recommendations from individuals familiar with the candidate and his work.
3. A proposed program of study.
4. Acceptance of the individual by the head of the department in which the Fellowship will be held.
5. A statement that he or she will serve full time if awarded a Fellowship. A small amount of time (10 to 15 per cent) may be spent in course work or participation in teaching, the latter purely on a voluntary basis.

The Upjohn Scholar of The Endocrine Society

The Council of The Endocrine Society has established a category of Scholars. These grants have been made available through the generosity of the Upjohn Company, and will be awarded to an established investigator or teacher in the field of endocrinology who wishes to extend the opportunities for work either in this country or abroad.

The award will not exceed $2,500 annually and will be granted on the basis of proposals submitted by the applicant. Such applications should include estimated financial needs. The funds may be used for travel, maintenance and other expenses.

THIRTEENTH POSTGRADUATE ASSEMBLY IN ENDOCRINOLOGY AND METABOLISM

Under the Co-Sponsorship of The Endocrine Society and the National Institutes of Health
Bethesda, Maryland
Oct. 2–6, 1961

A comprehensive review of clinical endocrine problems and current research activity in these areas will be presented.

For further information write to:

Dr. Roy Hertz
National Institutes of Health
Building 10
Bethesda 14, Maryland.

The fee will be $100.00 for physicians with a reduction to $30.00 for Residents and Fellows. Enrollment limited to 100.

VIII INTERNATIONAL CANCER CONGRESS
APPLICATIONS AND TRAVEL ALLOTMENTS

Announcement

The VIII International Cancer Congress will be held in Moscow, USSR, 23–28 July 1962, under the sponsorship of the International Union Against Cancer. Papers are invited on experimental or clinical aspects of cancer or on cancer control. These papers must not have been published or presented prior to 23 July 1962. The deadline for submission of abstracts is 1 November 1961, the original and two copies to be sent to Moscow, and an original and five clearly legible copies to the USA National Committee on the International Union Against Cancer.

Special facilities will be available for motion pictures and technical exhibits, a brief description of which should be submitted in the same style as abstracts of papers.

Deadline for registration without late fee is 1 April 1962. An information brochure and application forms for registration, submission of papers, etc., are available on request to either of the following:

Prof. L. M. Shabad, General Secretary
Soviet National Organizing Committee
Academy of Medical Sciences of the USSR
14 Soljanka
Moscow, USSR

Dr. Harold F. Dorn, General Secretary
International Union Against Cancer
National Institutes of Health
Bethesda 14, Maryland

Travel allotments will be available to a limited number of scientists and physicians residing in the USA who may require such assistance. The grant will be designed to cover round trip economy jet fares, an 11-day per diem allowance, and reimbursement for the $30 registration fee.

Applications for travel allotments should be submitted in sextuplet in the form of letters giving information on age, training, academic or pro-

fessional title, institutional affiliation, and the titles of the five most pertinent publications in cancer or related fields within the last five years, with reprints of the work the applicant considers to be his major contribution of the last five years. These letters should be countersigned by the department director or administrative officer. Applicants for travel funds who are submitting papers for presentation at the Congress must include six copies of an abstract (not to exceed 250 words) of each such paper, as noted above. An applicant not planning to present a paper at the Congress should include six copies of an abstract of major current investigative work. Those invited to participate in symposia or other meetings sponsored by the IUAC should give full particulars, indicating what additional expenses, if any, will be involved. Such letters and abstracts must be submitted in sextuplet before 1 November 1961 to the Chairman, USA National Committee on the International Union Against Cancer, 2101 Constitution Ave., N. W., Washington 25, D. C.

Applicants will be responsible for their own passports, visas, registration, travel arrangements, and hotel reservations.

INTER-SOCIETY CYTOLOGY COUNCIL, INC.

Inter-Society Cytology Council, Annual Scientific Meeting, Hotel Peabody, Memphis, Tennessee, November 2, 3, 4, 1961. Dr. Paul A. Younge, Secretary-Treasurer, 1101 Beacon Street, Brookline 46, Massachusetts. Everyone interested in cytology is welcome to attend.

INTERNATIONAL CONGRESS ON HORMONAL STEROIDS

Milano, Italy—May, 1962

The scientific program includes: a) Six Symposia at which lectures will be given by prominent invited speakers, b) Sessions of short communications, c) Private Scientific Meetings and Round Table Discussions.

The following 6 Symposia are planned: 1) new steroids with hormonal-like activities, 2) pathways of synthesis and metabolism of hormonal steroids, 3) control of synthesis and release of steroid hormones, 4) mechanism of steroid action, 5) substances which affect the synthesis and action of steroid hormones, 6) non-hormonal activities of steroids.

The Symposium lectures will be of 20 minutes' duration, followed by 10 minutes' discussion.

The speaking time for short communications will be 10 minutes. A number of lectures rooms will be available for Private Scientific Meetings and Round Table Discussions.

Please write to the Secretaries for inclusion of special subjects in the final program.

Official languages of the Congress will be English, French and Italian.

Simultaneous translation from and into each of these languages will be

provided for Symposium lectures and discussions.

All members of the congress will receive abstracts of lectures and of short communications at time of registration.

The Proceedings of the Meeting will be published in bookform after the Congress.

For further information, application forms and forms for submission of papers please write to the Secretaries.

SYMPOSIUM ON RECENT DEVELOPMENTS IN RESEARCH METHODS AND INSTRUMENTATION

Announcement

11TH ANNUAL INSTRUMENT SYMPOSIUM AND
RESEARCH EQUIPMENT EXHIBIT

OCTOBER 9 THROUGH 12, 1961

NATIONAL INSTITUTES OF HEALTH, BETHESDA 14, MARYLAND

Instrument Symposium

Committee on Symposium on Recent Developments
in Research Methods and Instrumentation

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DR. JULIUS SENDROY, JR.
Naval Medical Research
Institute
Bethesda 14, Maryland

Executive Secretary

MR. JAMES B. DAVIS
National Institutes of Health
Bethesda 14, Maryland

Primary topics for discussion on the scientific program include: (1) Applied Gas Chromatography; (2) Factors Influencing Interpretation of Spectra; (3) Electron Magnetic Resonance; (4) Thermogravimetric Analysis; (5) Electron Probe Analysis; (6) Application of Physiological Instrumentation to Clinical Problems; and (7) Optical Rotatory Dispersion.

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National Institutes of Health
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Examples of the latest types of research equipment will be exhibited by instrument manufacturers.

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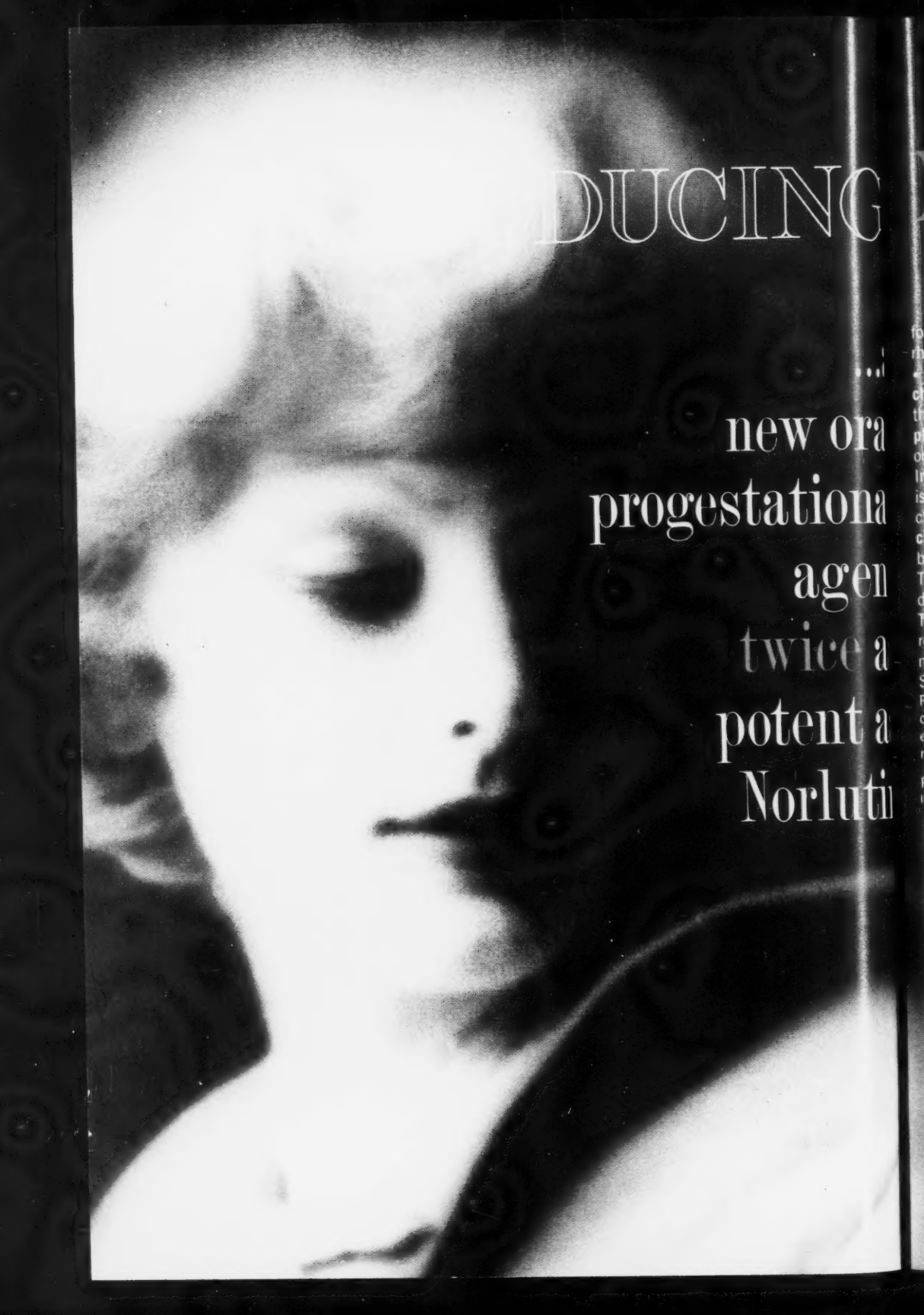
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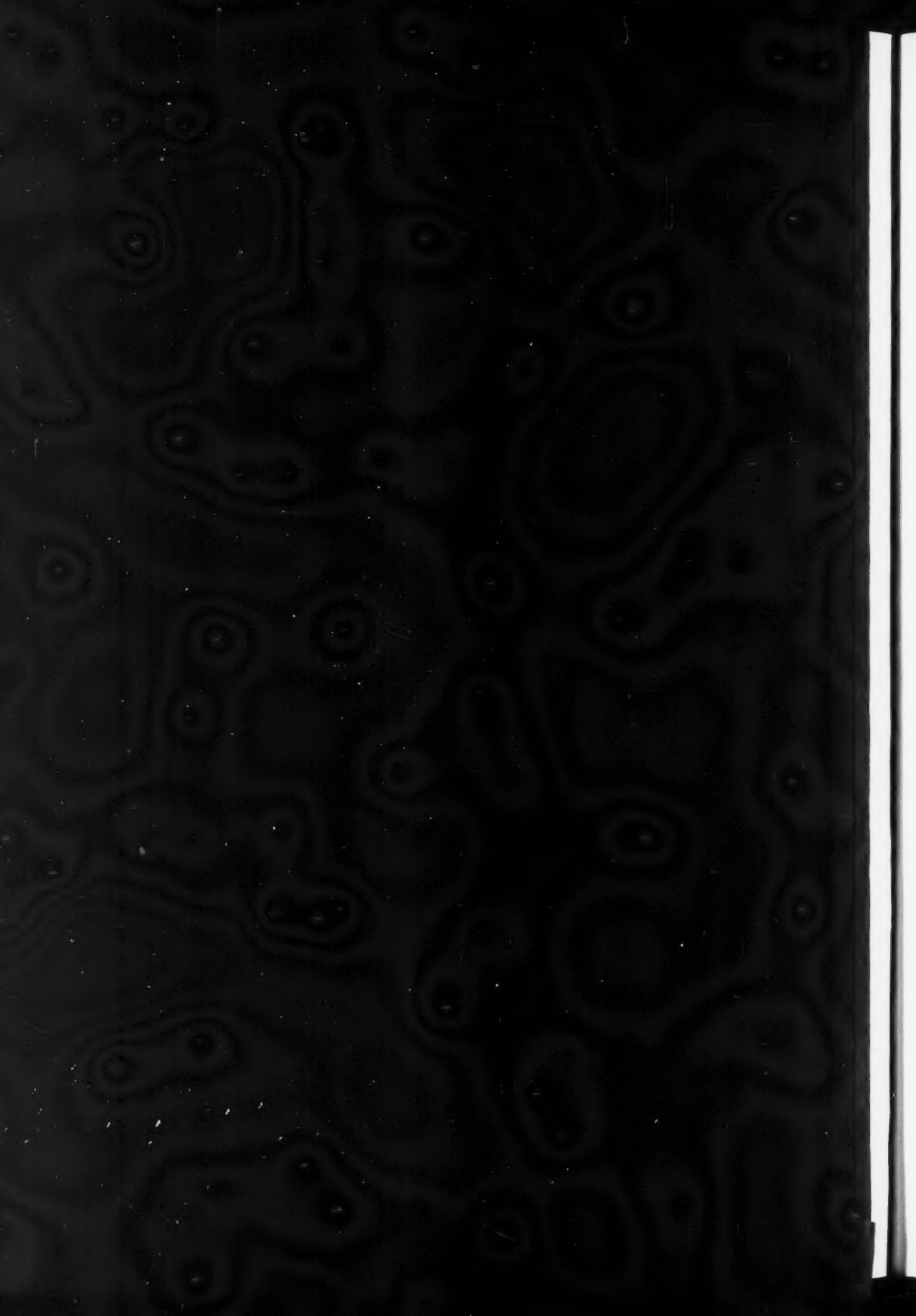

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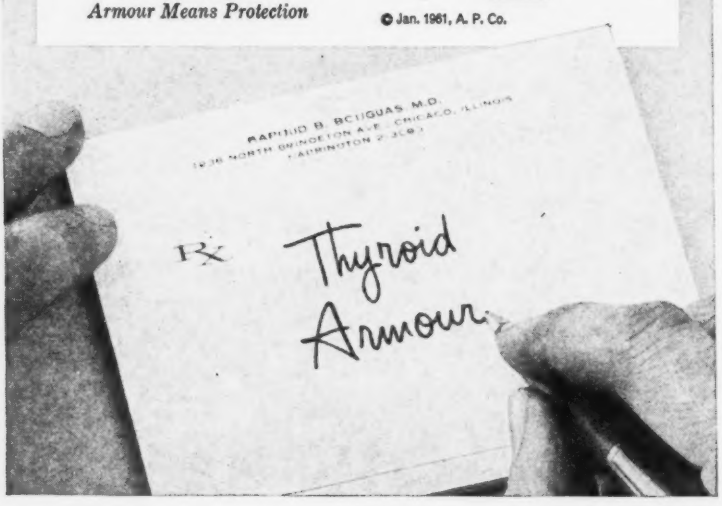

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